

THE BIOCHEMICAL JOURNAL

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By R A Peters and R W Wakelin

Volume 40 (1946), No 4, p 516, col 2, line 9

for fraction *read* reaction

Porphyrimuria in Rats Fed Oxidized-casein a Preliminary Communication

By C E Dent and C Rimington

Volume 41 (1947), No 2, p 260, col 2, lines 35-36 (References)

for J biol Chem 128, 399 *read* J biol Chem 141, 871

Obituary Notice, Frederick Gowland Hopkins, 1861-1947

Volume 42 (1948), No 2, p 169, col 1, line 13

for one *read* three

New Zealand Fish Oils 4 Observation on the Oil Content
of Fresh Water Eels

By F B Shorland and J Russell

Volume 42 (1948), No 3, p 481, col 2, line 15

for Table 2 *read* Fig 2

The Isolation and Chemical Properties of Trichothecin, an Antifungal Substance from *Trichothecium roseum* Link

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(Received 4 June 1948)

Antagonism between *Trichothecium roseum* Link and various fungi pathogenic to plants has been reported by Whetzel (1909), Boning (1933), Koch (1934) and Greaney & Machacek (1935). Culture filtrates of *T. roseum* have been shown to inhibit germination of *Botrytis allii* conidia (Brian & Hemming, 1947). A preliminary account of the isolation and properties of trichothecin, an antifungal compound from *Trichothecium roseum*, has been given by Freeman & Morrison (1948). The present paper gives a detailed description of the isolation and properties of the antifungal compound.

EXPERIMENTAL

Strains of *Trichothecium roseum*

The strains used in this work were typical cultures of *T. roseum* and were isolated in the laboratory mainly from dead wood collected locally. The strains are distinguished by their laboratory catalogue numbers. The cultures are described in detail by Freeman & Morrison (1949).

Isolation of trichothecin

Medium. The following medium was used for production of trichothecin. It is based on the formula of Czapek (as modified by Dox, 1910), ammonium tartrate has been substituted for NaNO_3 as source of N, and corn steep liquor has been added as a supplementary nutrient (Freeman & Morrison, 1949).

Ammonium tartrate	2.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
K_2HPO_4	1.0 g
KCl	0.5 g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01 g
Glucose	50 g
Corn steep liquor	10 ml
Water to	1000 ml

Crude commercial glucose ('glucose chips') was found to be satisfactory for this purpose. The medium (800 ml) was sterilized by autoclaving in 'Glaxo' bottles (Clayton, Hems, Robinson, Andrews & Hunwicke, 1944). The reaction was adjusted to pH 5.0. Each bottle was inoculated with 1 ml of a spore suspension from a test-tube slope culture (about 7 days old) of *T. roseum* F 227 on beer-wort agar medium. The cultures were incubated at 25° for 28 days in darkness.

Extraction and isolation. At the time of harvesting, trichothecin concentration in the culture filtrates was determined

by the *Penicillium digitatum* spore germination method (Freeman & Morrison, 1949). The cultures were filtered, the mycelium washed with a little water and the filtrates and washings from a batch of bottles (usually about 40) combined for chloroform extraction. The filtrates were clear and brown in colour, they were readily filtered through paper. The filtrate in lots of 1 l was twice extracted with chloroform (200 ml). The extracted liquor was free from antifungal activity as determined by the *P. digitatum* spore germination method. The chloroform extracts were combined and evaporated to dryness under reduced pressure. The dried extract from 16 l of culture filtrate was obtained as a brown gummy solid (2.165 g) which was fractionated as shown in Fig. 1, further details of the chromatographic technique are given below.

In one experiment, the combined extracts from 84 l (7.885 g) were dissolved in ether (25 ml) and poured on to a column (30 cm long \times 2.2 cm diam) of activated alumina. The column was developed with ether and the eluate collected in 100 ml fractions. Practically the whole of the antifungal substance was contained in fractions 1, 2 and 3, which gave dry weights of 1.392, 3.196 and 0.433 g respectively. The fractions were dissolved in a 9 l (v/v) mixture of light petroleum (b.p. 60–80°), and chloroform (100 ml) on a steam bath. After 18 hr at 20°, crystalline precipitates of an inactive compound (II) were separated off. On evaporation to dryness, the combined filtrates gave an almost colourless syrup (4.292 g). The latter was dissolved in carbon tetrachloride (25 ml), and poured on to an alumina column similar to that described above. The chromatogram was developed with a mixture of chloroform and carbon tetrachloride (1:1) and the eluate collected in 50 ml fractions. On evaporation to dryness, the trichothecin fractions crystallized as fine, colourless needles (3.472 g). Recrystallization from hot light petroleum (b.p. 60–80°), gave pure trichothecin (3.015 g), m.p. 118° (all melting points are corrected). Yield about 36 mg/l of culture filtrate. Details of the recovery from three batches are given in Table 1.

Properties of trichothecin

Biological. The antifungal activity of trichothecin is exhibited against Fungi Imperfecti, Zygomycetes and Ascomycetes. The growth of each of some 25 species belonging to the above classes was in some degree inhibited (Freeman & Morrison, 1949). At a concentration of 400 mg/l and pH 7.0, trichothecin was inactive against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*.

Chemical and physical. Trichothecin crystallizes from light petroleum in long fibrous needles, m.p. 118°. It is readily soluble in chloroform, ethanol, acetone and benzene,

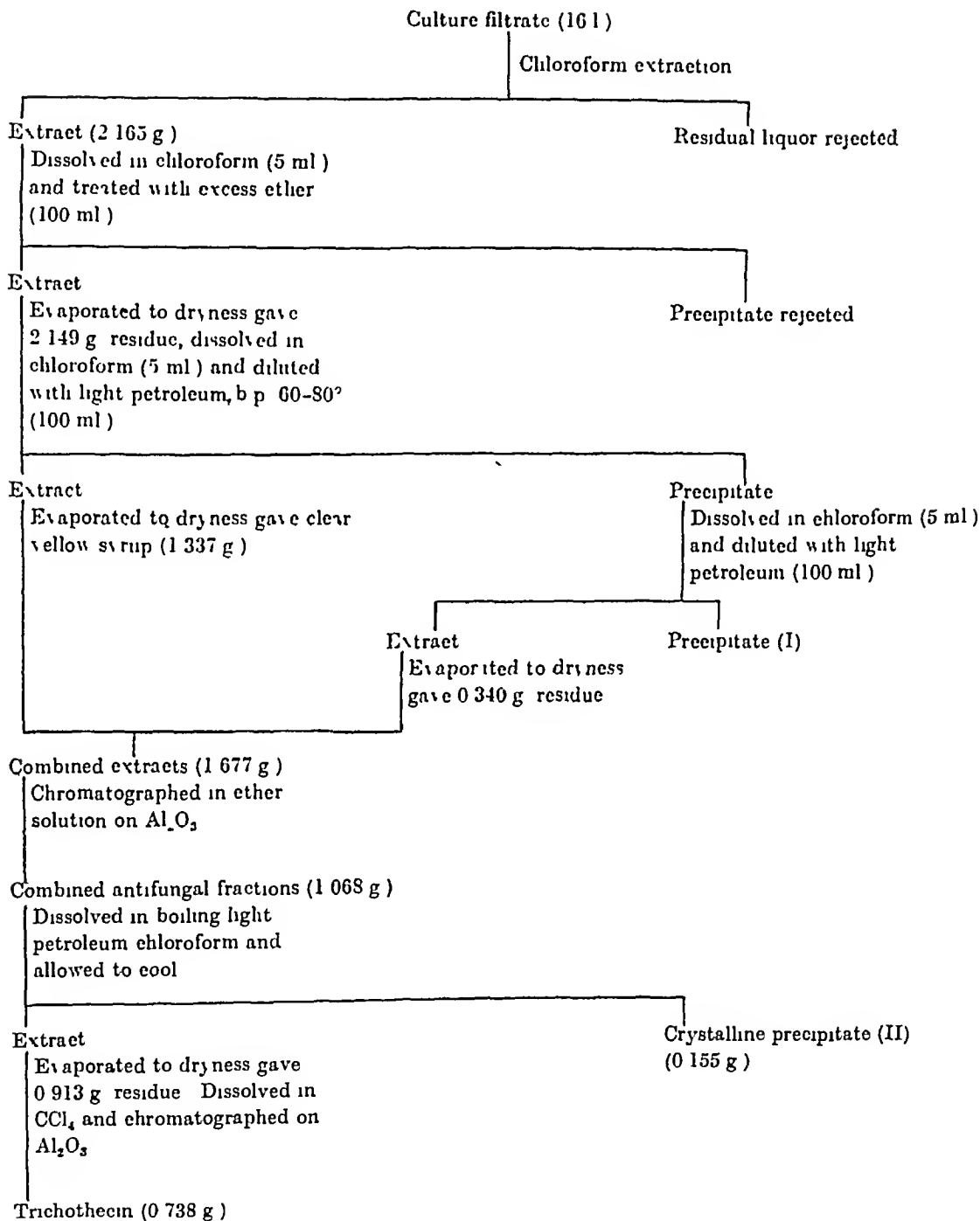


Fig 1 Isolation of trichothecin

Table 1 Summary of data on recovery of trichothecin from batches of culture filtrate

Batch no	Time of incubation (days)	Vol of culture filtrate (l)	Dry wt of mycelium (g)	Trichothecin assay (mg/l)	Total trichothecin calcd from assay (g)	Wt of chloroform extract (g)	Wt of trichothecin isolated (g)	Trichothecin yield	
								From culture filtrate (mg/l.)	Assay (%)
1	14	84	—	47	3 95	15 88	3 01	35 8	76 1
2	28	78	715	62	4 84	21 23	3 20	41 0	66 1
3	28	78	939	64	4 99	23 98	3 42	43 8	68 5

slightly soluble in light petroleum and very slightly soluble in water (400 mg/l. at 25°). It is optically active, $[\alpha]_D^{18} +44^\circ$ (c, 1 in chloroform). The compound contains no halogen, S or N. Micro analyses (Weiler and Strauss). Found C, 68.5, 68.7, 68.8, H, 7.2, 7.3, 7.4, mol. wt. (Rast), 278. $C_{15}H_{20}O_4$ requires C, 68.2, H, 7.5, mol. wt., 264. $C_{15}H_{18}O_4$ requires C, 68.7, H, 6.9%, mol. wt. 262.

There is no evidence of the presence of free carboxyl, hydroxyl, alkoxy or aldehydic groups in the molecule. The presence of a ketonic group is indicated by reactions with hydroxylamine, semicarbazide and 2,4-dinitrophenylhydrazine. The oxime and semicarbazone were not well characterized crystalline derivatives.

Trichothecin 2,4-dinitrophenylhydrazone Trichothecin (0.13 g, 0.5 mmol.) was dissolved in ethanol (2 ml.) and mixed with 2,4-dinitrophenylhydrazine (0.1 g.) which had been dissolved in conc. H_2SO_4 (0.3 ml.) and diluted with ethanol (2 ml.). After 18 hr. at 20° the mixture was diluted with 2N- H_2SO_4 (10 ml.) and extracted with ether (20 ml.). The extract was washed with 2N- H_2SO_4 , then several times with water and dried over anhydrous Na_2SO_4 . After filtration and concentration to 10 ml. the solution was chromatographed on a column of activated alumina. The main fraction (orange band) was eluted with ether and recrystallized from industrial spirit as orange needles (0.165 g.), m.p. 191–193°. A second recrystallization from ethanol gave the *hydrazone*, m.p. 200°. Found C, 57.8, 58.6, H, 5.3, 5.5, N, 10.6, 12.6. $C_{21}H_{24}O_7N_4$ requires C, 56.9, H, 5.4, N, 12.6%.

The presence of ethylenic unsaturation was indicated by reduction of $KMnO_4$ in a cold acetone solution of trichothecin. There was, however, no reaction with Br_2 in carbon tetrachloride. Microhydrogenations of trichothecin in acetic acid solution were carried out in the presence of Pd black catalyst and Adams's platinum oxide catalyst. With the Pd catalyst, trichothecin (3.255 mg.) absorbed 0.570 ml. H_2 at 744 mm/18°, absorption was complete in 35 min. Number of double bonds = 1.89. With the Adams platinum oxide catalyst, trichothecin (3.240 mg.) absorbed 0.600 ml. H_2 at 742 mm/20°, absorption being complete in 20 min. Number of double bonds = 1.98. (The authors are grateful to Prof. J. W. Cook, in whose laboratory these determinations were made.) Two molecules of H_2 were absorbed in the presence of each of the catalysts. This is consistent with the presence of conjugated carbonyl and ethylenic groups.

Trichothecin (1.00 g.) in absolute ethanol (50 ml.) was catalytically reduced at 760 mm/20° in the presence of Adams's platinum oxide catalyst (0.02 g.). Hydrogenation was complete after 35 min. when 220 ml. of H_2 (119% of the theoretical) had been absorbed. The product (1.021 g.) was a colourless syrup, which did not crystallize.

Determination of methyl groups attached to carbon (Weiler and Strauss) gave $(C)CH_3$, 16.4, $C_{12}H_{11}O_4(CH_3)_3$ requires 17.0%. Methoxyl determination gave OCH_3 , 0.24%. Theoretical for 1 OCH_3 /mol. ($C_{15}H_{20}O_4$), 11.8%.

Alkaline hydrolysis of trichothecin Trichothecin (0.100 g.) was heated under reflux with ethanolic KOH (20 ml., approx. 0.1N). After 3 hr. the solution was cooled, diluted with water (10 ml.) and titrated with 0.1N HCl using phenolphthalein as indicator. The titration difference was 3.75 ml. 0.1N acid, which corresponds to an equivalent of 266 (theoretical for 1 potential carboxyl group per molecule ($C_{15}H_{20}O_4$), 264).

Excess acid was added and the bulk of the ethanol removed by distillation under reduced pressure. The residue

was diluted with water (10 ml.) and twice extracted with chloroform. The extract was washed, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue (0.091 g.) was a pale yellow syrup which crystallized on cooling. On recrystallization from a mixture of benzene and light petroleum, b.p. 60–80°, it was obtained as colourless prisms, m.p. 181°. The product was readily soluble in chloroform, and only slightly soluble in ether. The reactions of the compound showed that a ketonic group was present. Found C, 67.8, 67.9, H, 7.5, 7.3, mol. wt. (Rast) 212, 299. $C_{15}H_{20}O_4$ requires C, 68.2, H, 7.5, mol. wt. 264.

Oxidative degradation Attempts to obtain characteristic degradation products by oxidation with various reagents such as $KMnO_4$, $K_2Cr_2O_7$, $KBrO_3$, lead tetra-acetate and SeO_2 have so far been unsuccessful. Although oxidation usually took place, the products resisted attempts at crystallization or purification. Further work is being done on these lines.

Stability of trichothecin The reaction of aqueous solutions of trichothecin (20 mg./l.) was adjusted to pH 1 and 10 by addition of HCl and NaOH, respectively. After suitable intervals, the solutions were neutralized and trichothecin concentration determined by the *Penicillium digitatum* spore-germination method. There was no significant loss of activity at 20° after 48 hr. at pH 1 and 10. At pH 12, the antifungal activity was completely destroyed in 6 hr. at 20°. The rate of inactivation of trichothecin at pH 12 and 20° corresponded to that of a first order reaction in which k was approximately 0.01. Aqueous solutions of the antifungal substance at pH 7 showed no detectable loss of activity after 1 hr. at 100°.

Cavallito & Bailey (1944) reported that a number of antibiotics such as gliotoxin, patulin and penicillic acid were inactivated by cysteine. Addition of cysteine (30–1000 mg./l.) to a solution of trichothecin (0.625 mg./l.), which permitted germination of 5% of *P. digitatum* spores in 18 hr., had no effect on the number of spores which germinated.

Ultraviolet absorption spectrum The following main absorption bands were observed with chloroform or hexane solutions of trichothecin, (i) broad shallow band at 334 $m\mu$, with molar extinction coefficient ϵ , 44, (ii) intense band at 220 $m\mu$, ϵ , 10^4 . Ethanol solutions exhibited the following absorption bands, (i) broad shallow band at 325 $m\mu$, ϵ , 37, (ii) intense band at 230 $m\mu$, ϵ , 10^4 .

The alkaline hydrolysis product from trichothecin (in chloroform or ethanol) exhibited the following absorption bands, (i) intense band at 230 $m\mu$, ϵ , 10^4 (based on a mol. wt. of 262), (ii) shallow, broad band at 339 $m\mu$, ϵ , 49. The position of the bands was not altered by a change of solvent from ethanol to the less polar solvent chloroform. Band (i) is attributed to the presence of an ethylenic linkage and band (ii) to a ketonic group.

Infrared absorption spectrum The infrared absorption spectrum showed a group of characteristic bands at 815, 847, 970, 1080, 1180 and 1290 cm^{-1} . These should be useful in characterizing the compound. C—H bond bendings were shown at 1367 and 1460 cm^{-1} . Strong absorption in the region 1645–1700 cm^{-1} indicated the presence of carbonyl groups and an ethylenic group. Separate bands occurred at 1645 cm^{-1} (unsaturation), and 1677 and 1698 cm^{-1} (carbonyl groups). C—H bond stretching, near 2900 cm^{-1} , was shown, but there was no indication of the presence of hydroxyl groups. There was no trace of benzenoid absorption.

DISCUSSION

It is concluded that trichothecin is responsible for the antifungal properties exhibited by culture filtrates of *Trichothecium roseum*. Trichothecin isolated by the process described accounts for about 70% of the fungistatic activity of the culture filtrates as determined by the *Penicillium digitatum* spore germination method (cf Table 1). None of the fractions obtained in the fractionation described in Fig 1 exhibited significant antifungal activity apart from the trichothecin-containing fractions. Crystalline products have been isolated from fractions I and II and will be described in a later paper, they were devoid of antifungal or antibacterial activity. In the chromatographic separations, only the trichothecin-containing fractions inhibited germination and growth of *P. digitatum*.

The molecular structure of trichothecin is not yet known. The analytical data are in good agreement with the molecular formulae, $C_{15}H_{18}O_4$ and $C_{15}H_{20}O_4$, of which the latter is preferred. There is at present insufficient evidence to decide between these alternatives. The molecule has been shown to contain one ketonic group, one ethylenic group and three methyl groups attached to carbon. Other functional groups, such as free carboxyl, hydroxyl, alkoxy and aldehydic groups, have been shown to be absent. On hydrolysis with ethanolic potassium hydroxide, trichothecin combined with one equivalent of the alkali, indicating the liberation of one carboxyl group. It has not been possible to isolate the corresponding acid, since on acidification a neutral compound, probably isomeric with trichothecin, was formed. This compound, which had no antifungal properties, differed from trichothecin in containing unconjugated ketonic and ethylenic groups.

The ultraviolet absorption spectrum of trichothecin contained two main bands which corresponded closely with the bands in the spectra of unsaturated aldehydes and ketones referred to by Morrison (1947) as the *R* and *K* bands. In conjugated systems of this type the position of the bands is influenced by the polarity of the solvent. Change from a relatively non-polar solvent such as hexane or chloroform to a polar solvent such as ethanol results in a shift of the *R* band to shorter wave lengths whilst the *K* band moves towards the longer (Morrison, 1947). This was the case with the trichothecin spectra, which indicates that the ethylenic and carbonyl groups in the molecule are conjugated with respect to each other.

The alkaline hydrolysis product, on the other hand, had a spectrum in which the corresponding bands were not altered in position by a change in solvent from chloroform to ethanol. These bands are attributed to the presence of unconjugated ethylenic and ketonic groups. The chemical evidence denoted the presence of a ketonic group. The effect of alkali

is interpreted as due to the opening of a lactone ring with formation of the potassium salt. The acid equivalent of the alkali used in the reaction corresponded to the formation of one carboxyl group per molecule. On reacidification, a new neutral compound was formed, isomeric with trichothecin, in which unconjugated ketonic and ethylenic groups were present.

Infrared absorption-spectrum observations confirmed the conclusions drawn from the chemical and ultraviolet data that carbonyl groups and an ethylenic group were present. The infrared absorption data also indicated the absence of hydroxyl groups and benzenoid structures. The presence of carbon-methyl groups and ether linkages was suggested.

Trichothecin has been shown to be fairly stable at pH 1-10 at ordinary temperatures. At pH 12, hydrolysis, following a unimolecular reaction course, took place with virtually complete loss of antifungal activity in 6 hr at 20°. The inactivation was irreversible. At pH 7.0, trichothecin was not destroyed in 1 hr at 100°. These data suggest that the antifungal compound may persist sufficiently long under natural conditions to influence the growth of certain fungi and lead to the antagonism between *Trichothecium roseum* and other fungi mentioned in the introduction.

The antifungal activity of trichothecin does not appear to depend upon its intervention in a metabolic reaction involving SH groups, since its activity is unaffected by the presence of a large excess of cysteine. Further work on the structure of trichothecin is proceeding.

SUMMARY

1 The isolation of trichothecin from the culture filtrate of *Trichothecium roseum* is described. The antifungal compound was extracted with chloroform and purified by fractional precipitation and chromatographic separation on alumina.

2 Trichothecin crystallized from light petroleum in long fibrous needles, *m.p.* 118°, $[\alpha]_D^{25} + 44^\circ$ (c, 1 in chloroform). The analytical data were consistent with the molecular formula $C_{15}H_{18}O_4$ or $C_{15}H_{20}O_4$.

3 The compound was a neutral, unsaturated ketone in which the carbonyl and ethylenic groups were conjugated.

4 Hydroxyl and alkoxy groups were shown to be absent. The presence of three carbon-methyl groups per molecule has been established.

5 Trichothecin was found to be relatively stable in acid solution and at pH 10, but at pH 12 hydrolysis took place with liberation of a carboxyl group, subsequent acidification led to formation of an inactive neutral ketone.

We wish to express our thanks to Dr N. F. H. Bright for the ultraviolet absorption data, to Mr K. S. Tetlow for the infrared absorption data, and to Mr A. J. Bailie and Miss A. McCann for their assistance in the preparation of the trichothecin used in this work.

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The Nucleoprotein Content of Fibroblasts Growing *in vitro*

4 CHANGES IN THE RIBONUCLEIC ACID PHOSPHORUS (RNAP) AND DEOXYRIBONUCLEIC ACID PHOSPHORUS (DNAP) CONTENT

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(Received 30 April 1948)

In the earlier papers of this series (Davidson & Waymouth, 1943, 1945, 1946) an account has been given of the conditions under which it is possible to record an increase in the nucleic acid content of chick-heart fibroblasts growing *in vitro*. Using the roller-tube technique of Willmer (1942), appreciable increases in the total nucleic acid phosphorus (NPP) of the fibroblasts have been obtained when the cultures were planted in fowl plasma and allowed to grow in chick embryo extract. Defatted chick-embryo extract and fowl plasma, either together or separately, produced smaller increases in nucleic acids measured over 48 hr periods, and led to a more rapid deterioration of the cultures (Davidson & Waymouth, 1946). Loss of nucleic acid was shown to occur over the 2 days following planting, and was greater for cultures in Tyrode solution than for those in embryo extract. However, the increase following addition of embryo extract to the cultures in Tyrode was as great 2 days later as in the case of cultures provided with embryo extract throughout. As a rule increases in NPP were greater for higher than for lower concentrations of embryo extract.

Schmidt & Thannhauser in 1945 published their method for determining the amounts of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in tissues (Schmidt & Thannhauser, 1945). This method has been adapted for estimating the phosphorus content of each acid after separation, and it has been possible to follow changes in these two components in the fibroblast cultures, and to investigate the relationship between growth and the total nucleic acid phosphorus (NPP), the ribonucleic acid phosphorus (RNAP) and the deoxyribonucleic acid

phosphorus (DNAP). Although Willmer (1942) and Cunningham & Kirk (1942) proposed that growth in tissue cultures could be measured by determining the NPP content of cultures at different stages of development, they did so before it had become clear that there were two types of nucleic acids in each cell, the more abundant one usually being the ribonucleic acid of the cytoplasm and nucleolus, the other, the deoxyribonucleic acid, being apparently always confined to the nucleus.

In the tissue-culture technique which we have employed, it is possible to select a basal starting level of NPP according to the number and size of the cultures employed. From the time when growth-promoting medium is added to the cultures, changes in the amounts of phosphorus from both types of nucleic acids can be measured at varying intervals of time. Since the tissues are in a resting state before addition of growth-promoting medium, and the production of new tissue is in abeyance, it is reasonable to suppose that if one nucleic acid is the precursor of the other, then it will be the first to appear in the growing cultures. In this work we have been able to show that an increase in the RNAP always precedes by relatively long intervals any increase in DNAP. If the assumption that the P content of the nucleic acids is invariable is justified (as has been questioned, Wiame, 1946), the synthesis of RNA appears to be established before that of DNA.

Our observations on the changes occurring in both the RNAP and the DNAP during growth of fresh embryo-heart cultures have confirmed the general findings of Brues, Rathbun & Cohn (1944) and have enabled us to find satisfactory conditions for growth

with the roller-tube method. Although with our original technique an increase in RNAP could always be obtained in growth-promoting media, a sustained and substantial rise in DNAP only occurred when the modification was introduced of cutting the individual explants small enough to reduce to a minimum loss of material by cell destruction at the centre.

METHODS

Fresh explants of 12 day chick embryo heart were grown in plasma clot in roller tubes (Willmer, 1942), the technique which has been employed in the earlier experiments of this series (Davidson & Waymouth, 1943, 1945, 1946). Each roller tube contained 24 pieces of tissue in three rows of eight and embedded in clots of 0.1 or 0.05 ml plasma per row according to the size of cultures employed. The growth promoting medium was either embryo extract alone or a mixture of embryo extract and serum. In each case 0.5 ml was allocated to each roller tube, and in experiments running over long periods, the fluid phase was renewed every 24 hr. Preliminary experiments showed that in control cultures in Tyrode solution the RNAP and DNAP fell to a steady level, which we have termed the resting state. In all the subsequent tests, the cultures were maintained for at least 20 hr in Tyrode solution before the addition of growth promoting medium.

Preparation of growth promoting media. Embryo extract (EE) was prepared from 12-day chick embryos by the method already described (Davidson & Waymouth, 1943) (total N, 50–90 mg/100 ml). In the preparation of the embryo extract-serum mixture (EES), 4 ml of the original concentrated extract and 2 ml of cockerel serum were diluted to 10 ml with Tyrode solution (final N concentration c. 60 mg/100 ml). Owing to the variations in plasma composition and in the concentration of embryo extracts prepared in the course of different experiments, comparisons of results are only made under the fairly uniform conditions of one particular experiment.

Estimation of RNAP and DNAP in the tissues. The two nucleic acid fractions were separated by the method of Schmidt & Thannhauser (1945), and the P content of each determined by the method of Berenblum & Chain (1938) as modified by Davidson & Waymouth (1943). It was possible to detect changes in the amount of P of the order of 0.1 μ g over the range of 0.1–8.0 μ g P, and determinations were carried out on the contents of one or two roller tubes according to the amount of tissue and plasma employed in the particular experiment.

The methods of extraction and separation of the nucleic acids were the same in every case. The fluid phase was discarded and the tube washed out with a small volume of 0.9% saline. Two ml of ice cold 10% (w/v) trichloroacetic acid (TCA) were added to each tube, and the tissues and plasma scraped off the sides of the tube. The contents were transferred to 15 ml graduated pyrex tubes, and centrifuged rapidly. The roller tube was washed out and the tissue extracted twice more with 2 ml portions of TCA, the whole operation being carried out within 20 min to avoid any loss of nucleic acid by hydrolysis, in this way, only acid soluble P was removed by the TCA treatment. Extraction of the lipid P was completed in a series of extractions using 80% and

absolute ethanol, two treatments with chloroform-ethanol (1:3) mixture at 70–80°, and finally ether. In most cases the extracts were discarded after centrifuging, and only in a few experiments were acid soluble and lipid P determined. The P in the dry powder resides mainly in the nucleic acids, since the amount of phosphoprotein in this tissue has been shown to be negligible.

For the separation of RNAP and DNAP, the residue was incubated at 37° overnight in the centrifuge tube with 1 ml of silica free N-NaOH, and the unhydrolyzed DNA separated from the hydrolyzed RNA by addition of 1.5 ml distilled water, 0.5 ml 2.5N HCl, and 0.6 ml of 30% (w/v) TCA. The DNA containing precipitate was spun down by centrifuging rapidly for 15–20 min, the supernatant liquid (the RNA fraction) was transferred to a short, wide necked, pyrex boiling tube. The precipitate was washed twice with 0.5 ml portions of 5% TCA, which were added to the supernatant fluid. After adding 0.3 ml 60% (w/w) HClO₄, the solution was evaporated at 100°, before transferring to an air bath in which complete oxidation of the tissue was carried out at a temperature of about 220°. The precipitate containing the DNA was oxidized in the same way with 0.2 ml of HClO₄. P was now estimated in both sets of materials, the results being corrected for reagent blanks, which were determined for each experiment.

Method of expressing results. In each experiment there were usually 14 roller tubes each containing 24 pieces of tissue in 0.3 or 0.15 ml of plasma, and 6 'plasma blanks' containing only the appropriate amounts of plasma, eluted by the addition of small pieces of heart tissue, which were subsequently removed. In most experiments, a pair of tissue tubes and a plasma blank were used for RNAP and DNAP determinations at zero time and at selected intervals during the growth period. When it was found that over certain periods there was little change in the plasma blanks, it was possible to dispense with intermediate samples and to correct some of the tissue estimations with mean or intermediate values.

It has not been possible to evolve a statistical basis for significance in the course of this work owing to variations in design and conditions from test to test. Moreover, there is an unknown error involved in the use of the plasma blank in the correction of the tissue figures, since an appreciable portion of the plasma clot is used up by the growing tissue as it extends its area, while there is no comparable alteration in the plasma tubes. Our practice of correcting for plasma blanks is likely to reduce the tissue values more than necessary, and so lead to an underestimation of the real increase in tissue P. It seems reasonable under these circumstances to consider these final increases in tissue P significant only when they equal or exceed the corresponding increases in the plasma blanks.

The initial values of both RNAP and DNAP in the tissue tubes and in the plasma tubes depend on the number and size of the cultures, and the composition of the plasma. Variations in the plasma blanks arise from the use of different batches of fowl plasma throughout these tests. The RNAP and DNAP contents per tube are produced by 24 relatively large pieces of tissue (12–15 mg fresh weight), while the contents per 2 tubes come from 48 much smaller pieces of tissue.

An examination of the RNAP figures (Table 1) shows that the real tissue increments (i.e. the values obtained after correction has been made for the corresponding plasma

blanks) are in every case considerably greater than the amounts of RNAP taken up by the plasma clots from the embryo extract or embryo extract-serum mixture. Both the tissue and plasma RNAP values, of course, increase as the duration of the growth period is extended, the amount of this increment depending on the concentration of the EE or EES. As these concentrations of extract are kept fairly uniform throughout each test, the change in the plasma blanks taken at different times is gradual, and their RNAP values do not fluctuate appreciably. The error involved in the actual determination of P by the method employed was of the order of $0.1 \mu\text{g } ^3\text{P}$, and it is considered that all the tissue RNAP increments shown in Table 1 are significant.

In the first group of tests in which the DNAP per tube of 24 cultures was measured, a significant increase is only found in tests 40, 49 and 50 (Table 2). In the others of this group there is very little change in the DNAP content, or there is a definite decrease which may be related to the relatively high content of the corresponding plasma blank, for which correction has already been made. Significant increases in DNAP are, however, obtained in the second group in which smaller explants (tests 56, 58, 59 and 62) are used, and while the plasma blank apparently falls in test 56, it increases considerably in tests 58, 59 and 62. The initial plasma value is unusually high in test 56.

RESULTS

The earlier work (Davidson & Waymouth, 1943, 1945, 1946) has shown that fresh chick-heart cultures lose an appreciable amount of their nucleic acid P (NPP) when they are maintained in Tyrode solution for 2 or 3 days after planting. This fall in total NPP was steep over the first 24 hr and more gradual over later periods. It also occurred when the cultures were maintained in embryo extract from the start, but in this case the drop in NPP content was not so large.

In the present work the changes which occur in the RNAP and DNAP contents of the cultures under the conditions described above have been investigated. Immediately after planting, the ratio RNAP/DNAP was found to lie between 2.2 and 2.8. If the cultures were maintained in Tyrode solution for 1 or 2 days, there was a loss of both RNAP and DNAP in amounts which left the ratio RNAP/DNAP much the same as in the freshly planted tissue, for example, for 12 tests the average figure for the ratio at this time was 2.25. This suggests, if anything, a slightly greater reduction in the RNAP than in the DNAP.

By the end of 20 hr in Tyrode solution, the removal of this easily lost nucleic acid was almost complete, and the tissues could be said to have reached their resting state (Fig 1). Since the loss involved both nucleic acids in the ratio of their occurrence in fresh tissue, it could be assumed to arise from the breakdown and washing out of cells damaged in the process of cutting the heart tissue.

The simultaneous fall in the RNAP and DNAP also occurred if embryo extract was used instead of

Tyrode solution in the period immediately following planting. That it was not so great as the fall occurring in Tyrode solution can be seen in Fig 1. If, after the first 20 hr, the Tyrode solution or extract was replaced by fresh extract, a visible increase in area of the cultures occurred, and it was accompanied by an increase in RNAP but not in DNAP. The final figure for RNAP is similar for both sets, although at the start of the second 21 hr test period the cultures which had been in embryo extract from the beginning had an appreciably higher RNAP content.

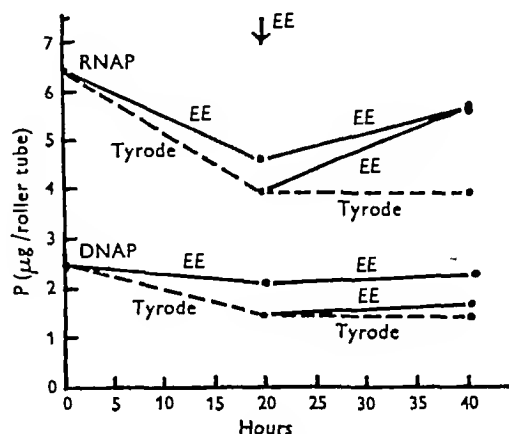


Fig 1 Changes in ribonucleic acid P (RNAP) and deoxyribonucleic acid P (DNAP) of chick-heart explants *in vitro*, using either Tyrode solution or embryo extract (EE) as the fluid phase. The figures for P represent the amounts found in the two nucleic acids in 24 pieces of tissue (planted in three rows of eight) per roller tube. The continuous line shows the changes which occur when the fluid phase was embryo extract, and the arrow indicates the time of the addition or renewal of EE. The broken lines show the changes in RNAP and DNAP occurring in Tyrode solution. The results at zero time refer to the cultures at the time of planting, at 20 hr those in Tyrode solution have reached their resting levels of RNAP and DNAP.

As a result of the experience gained in a few experiments of this nature, our practice in later experiments was to maintain the cultures for 2 days in Tyrode solution when changes in nucleic acid content were to be determined over relatively short periods. In experiments of long duration, lasting up to 7 days, it was considered sufficient to keep cultures for 20–24 hr in Tyrode solution before adding the growth-promoting medium.

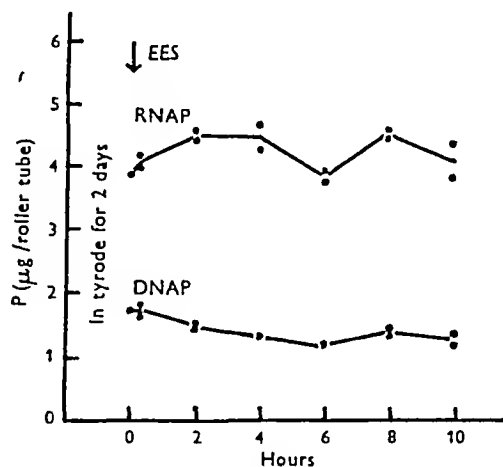
Changes in RNAP and DNAP content at short intervals over the first 48 hr in growth-promoting medium

The results of some of the preliminary experiments suggested that larger increases in NPP were obtained when the growth-promoting medium was a mixture of embryo extract (12-day chick embryo)

and fowl serum. This medium (EES) was employed throughout the series of tests to be described. The size of the individual cultures was relatively large,

original chick heart (15 μ g P/100 mg) (Davidson & Leshe, 1948), gives an average figure of 12–15 mg/24 tissue pieces.

In the first 12 hr in EES there was little or no visible change in the size of the cultures, and only a slight rise if any in RNAP (Fig 2). The DNAP remained unchanged, or fell during the same period.



Figs 2–8 The results are based on the contents of roller tubes, each containing 24 pieces of tissue, and have been corrected for plasma blanks. Most points are the mean of duplicate determinations. In some figures, RNAP/DNAP ratios are given at certain stages.

Fig 2 Changes in RNAP and DNAP in relatively large chick heart explants growing *in vitro* over the first 10 hr following the addition of a mixture of embryo extract and cockerel serum (EES). At zero hour, the cultures are in their resting state, and the arrow shows when the Tyrode solution was replaced by EES.

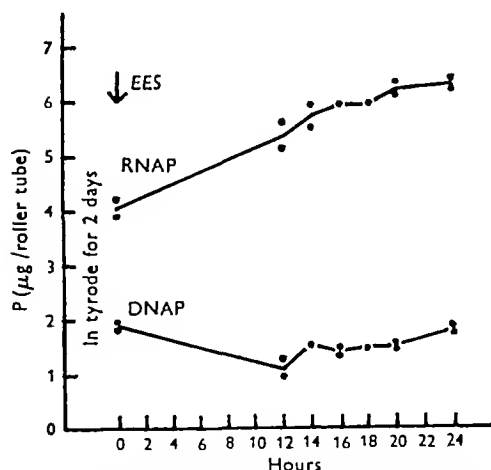


Fig 3 Changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro* over the first 24 hr, following the addition of EES. After planting, the cultures were maintained in Tyrode solution for 2 days, and the arrow shows when this was replaced by EES. The changes were followed at intervals of 2 hr in the second 12 hr period.

as compared with the pieces used in the final series of tests. An estimate of the weight of tissue in each roller tube at the start, based on the assumption that the DNAP content is the same as in the

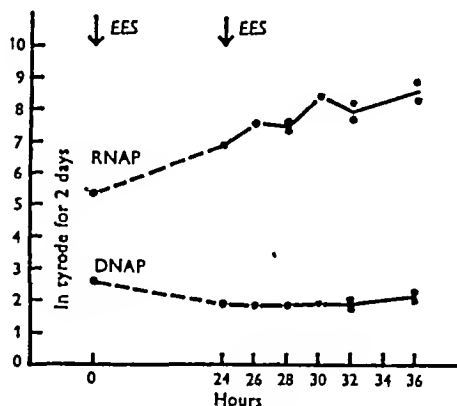


Fig 4 Changes in RNAP and DNAP in relatively large chick heart explants growing *in vitro* over the third 12 hr period following the addition of EES. The cultures were allowed to come to their resting state in Tyrode solution in 2 days, and the arrows show when the EES was added or renewed. The broken lines indicate that the time scale in the earlier stage has been condensed.

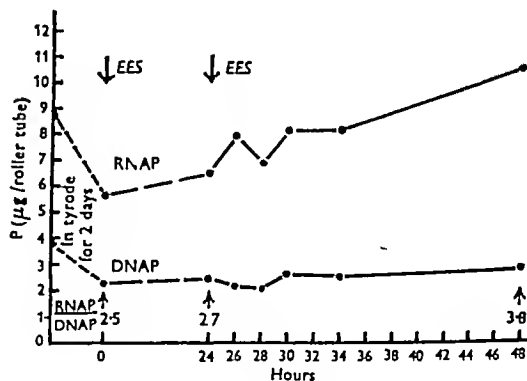


Fig 5 Changes in RNAP and DNAP in relatively large chick heart explants growing *in vitro* over a period of 48 hr following addition of EES. These changes were followed at intervals of 2 hr in the third 12 hr. The fall in RNAP and DNAP before zero hour occurs when the cultures are in Tyrode solution. The arrows show when EES was added or renewed.

The amounts of both nucleic acids, determined at intervals of 2 hr, showed regular fluctuations, which were unexplained. They were not, however, thought to be random experimental variations in view of the fact that this and similar curves are based on duplicate determinations, whose values in most cases lay close together. In the case of RNAP values, fluctuations of 0.5 μ g were considered significant if the duplicates agreed, since the corresponding

changes in the plasma blanks were only of the order of $0.1 \mu\text{g P}$. There was very good agreement between DNAP duplicates, and, in these, changes of $0.25 \mu\text{g P}$ can be considered significant.

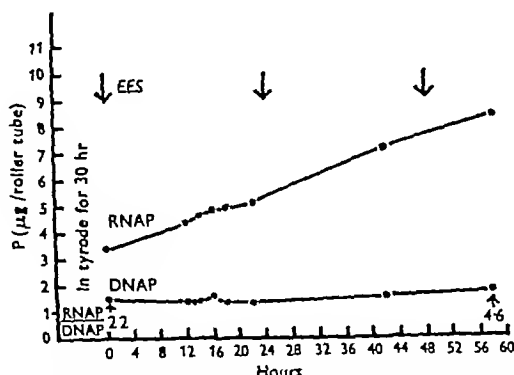


Fig 6 Changes in RNAP and DNAP in relatively large chick heart explants growing *in vitro* over 58 hr following the addition of EES. These changes were determined at intervals of 2 hr over the second 12 hr. After planting, the cultures were maintained in Tyrode solution for 30 hr, and the arrows show the times of addition or renewal of EES.

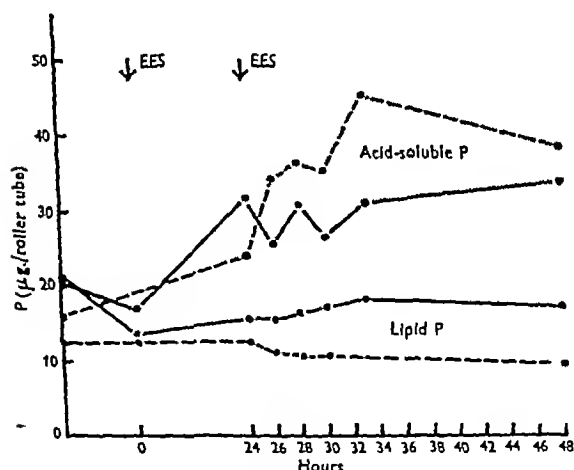


Fig 7 Changes in acid soluble P and lipid P in relatively large chick heart explants growing *in vitro* over 48 hr following the addition of EES. The conditions of the test are the same as in Fig 5, but the results for both the tissue tubes (unbroken lines) and the plasma blanks (broken lines) are given on separate curves, as the acid-soluble P is very much less in the tissue tubes than in the plasma tubes in the later stages. The arrows show the times of addition or renewal of EES.

A visible increase in the area of the cultures was observed in the first 24 hr in contact with the growth-promoting medium (as shown in Pl 1). In Fig 3 the RNAP increased appreciably over the same period, while the final DNAP content was much the same as at the start. The fall in the latter over the first 12 hr was confirmed, in this particular test it was accompanied by a large increase in the RNAP. In the second 12 hr period the RNAP seemed to increase evenly without any evidence of

the fluctuations which were characteristic of its behaviour in the earlier period. At the same time there was a definite increase in DNAP, but it is doubtful if the rise which occurred between 12 and 14 hr was of significance.

The marked rise in RNAP unaccompanied by a corresponding rise in DNAP calls for special comment. It was, of course, reflected in a rise in the RNAP/DNAP ratio from 2.2 to 4.2 over the 24 hr, a feature common to all the tests in which relatively large pieces of tissue were employed. This failure of

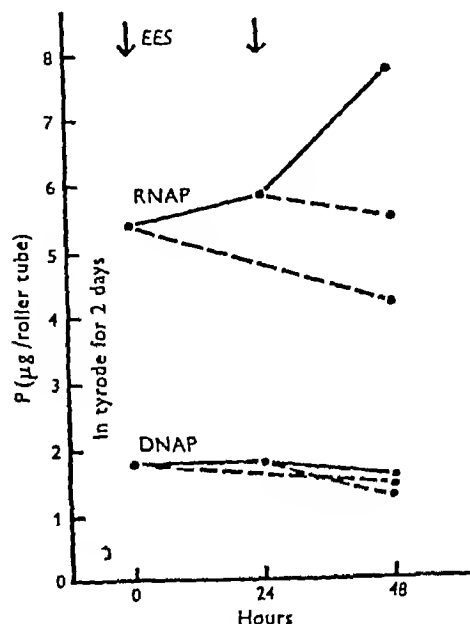


Fig 8 Effect of colchicine (1 in 4,000,000) on the changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro*, using EES as the fluid phase. The broken lines show the changes occurring when colchicine is present in the EES. After maintaining the cultures in Tyrode solution for 36 hr, EES or EES containing colchicine is added to the tubes at the times indicated by the arrows.

the DNAP to show any significant increase was perplexing at the time, in view of increases in RNAP and in the area of the cultures.

In Fig 4 are shown the results of a test on the changes occurring in the third 12 hr period. There was an unusually large fall in DNAP in the first 24 hr. Visible increase in area, continued, however, in the next 12 hr and the RNAP increased, although there was little change in DNAP. Again, regular fluctuations in RNAP were in evidence following the addition of growth-promoting medium. As similar patterns of RNAP changes were obtained in other tests they cannot be dismissed as random variations (see Fig 5). On the other hand, it is difficult to understand their significance, and it can only be recorded that the RNAP did not increase evenly over certain periods of growth.

In Fig 5 the initial drop in both fractions which occurred when the cultures were maintained in Tyrode solution for 2 days is again shown. In this test the changes over the first 24 hr were not irregular, the RNAP increased significantly, and there was a very slight rise in DNAP. The fluctuations following the second addition of EES showed the same features as before, with the rise in DNAP occurring in the later part of the first 12 hr period. Although small, these DNAP rises are considered significant, since the corresponding plasma-blank increases were very low or negligible in comparison with the increases in the DNAP of the tissues. At the end of 48 hr growth, the RNAP had increased 87% over its initial resting value, while the DNAP had increased by only 18%. By this time the cultures were very extensive (Pl 1C), and the rise in DNAP was, therefore, surprisingly small. The ratio RNAP/DNAP rose over the same time from 2.5 at the resting level to 3.8 after growth.

This increasing ratio, associated with a failure of the DNAP to show an appreciable increase, is a feature of another test, in which measurements were made over a 58 hr period (Fig 6). In this case, the RNAP increased by 166% as compared with a 16% increase in DNAP, or, expressed in terms of the ratio RNAP/DNAP, there was a rise from 2.2 to 4.6. Fig 7 shows the results of the determination of acid-soluble and lipid P which were made in the course of a test described above (Fig 5). As the acid-soluble P in the plasma blanks was higher than in the tissue tubes it was not possible to plot the corrected figures as in the case of RNAP and DNAP contents. Instead the values for tissue tubes and plasma blanks were both drawn on the same graph. The amounts of acid-soluble and lipid P increased in the tissues during growth of the cultures, but only the latter could be considered to increase in the tissue itself. Apparently, the acid-soluble P of the extract and plasma fell considerably in the tissue tubes during the same period.

Inhibition of growth of fibroblasts by colchicine

Colchicine in concentrations of 1 in 20–30 million has been shown to arrest mitosis in growing tissues *in vitro* (cf Bucher, 1940). This provided us with a means of confirming that increases in RNAP and DNAP in the presence of growth-promoting media were due to synthetic processes associated with the growth of new tissue.

The results of a typical experiment with colchicine are seen in Fig 8. The EES was divided into two 8 ml portions. To one was added 1 ml of a sterile Tyrode solution containing colchicine (1 in 400,000). The volume was made up to 10 ml with Tyrode solution. The 8 ml of EES for the control experiments were similarly diluted with Tyrode.

At the start of this test the majority of the roller tubes received the normal EES, and the remainder contained EES with colchicine (1 in 4,000,000). In the latter both the RNAP and DNAP values showed a distinct fall from the resting values at the end of 48 hr. There was, moreover, no visible sign of growth. In the others maintained in normal EES, good growth was observed, and the RNAP increased appreciably. When normal EES was replaced at the end of 24 hr by EES containing colchicine the RNAP and DNAP again fell during the next 24 hr and no further extension of area was observed.

The lower RNAP and DNAP of the cultures maintained in extract plus colchicine confirms that synthesis of these compounds is taking place in the presence of normal EES. In the case of the DNAP this is true even when no actual increase is observed in the growing cultures.

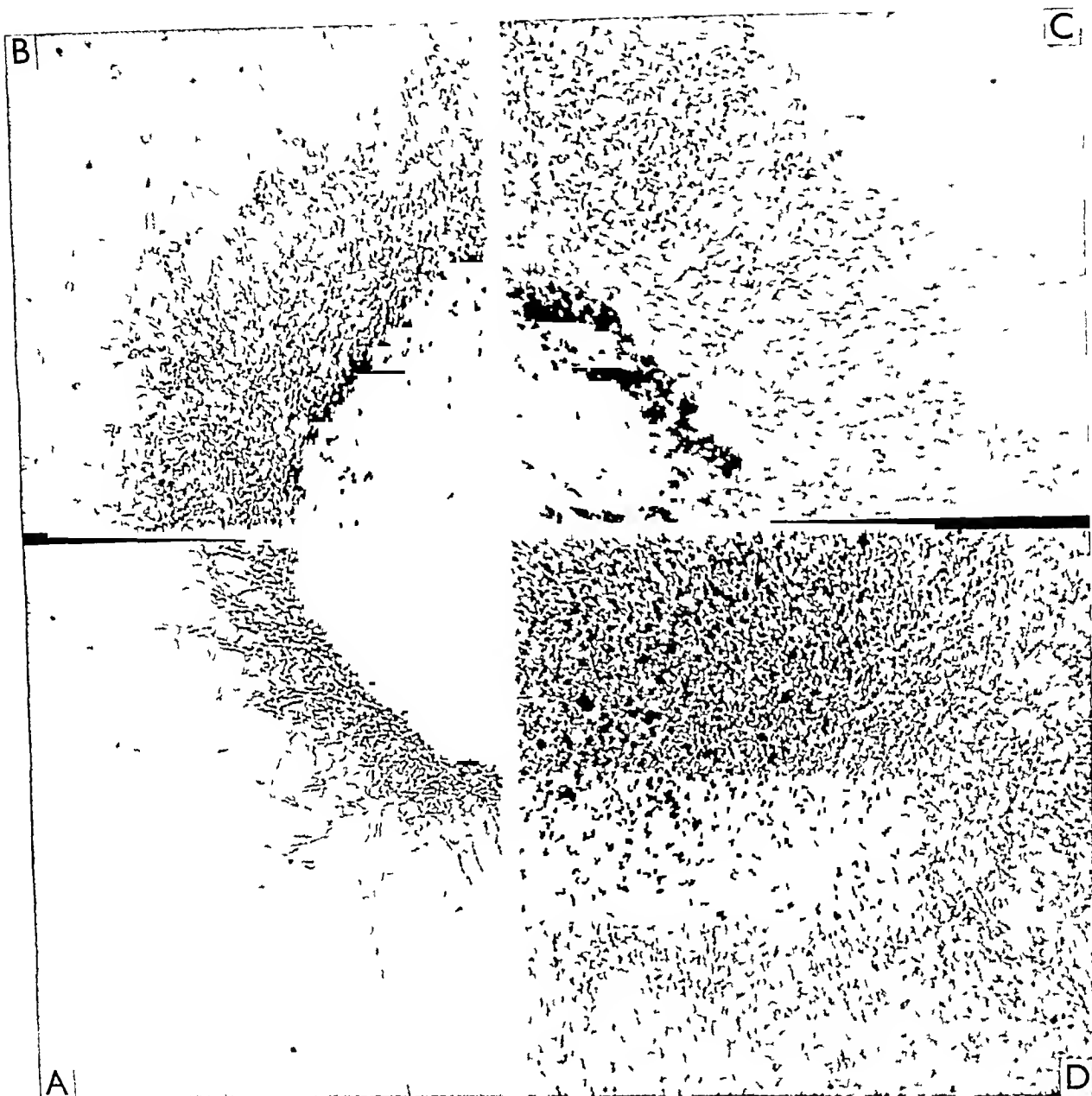
Changes in RNAP and DNAP over long periods of growth

In view of our failure over relatively short periods to obtain a rise in DNAP, commensurate with the rise in RNAP or the increase in area of the cultures, changes in RNAP and DNAP were followed over a period of 6–7 days. As in the earlier tests there was some indication that the rise in RNAP always precedes a rise in DNAP, it was thought at the outset that increases in DNAP might be greater at a later stage of development.

In the course of these tests of longer duration the EES had to be changed six or seven times, and care was taken to eliminate any sediment from the extract by centrifuging immediately before inserting each new portion of medium. If this was not done, the tubes were likely to become coated with precipitate, and the blanks to become unusually large.

Fig 9 shows the typical results obtained in a test of this nature, and in Pl 1 are photomicrographs showing the visible changes in the cultures. At the end of 120 hr, the cultures had spread to the limits of the plasma, and adjacent cultures were growing into one another. The central portions, which originally contained a kernel of compact heart tissue, were diffuse so that the core of each culture was translucent, and much larger than at the start. These visible changes were accompanied by a three-fold rise in RNAP, but once more there was hardly any increase in DNAP. The latter certainly rose slightly over 72–96 hr, but later when the diffusion of the original kernel of tissue was most noticeable, the DNAP fell. This behaviour was again reflected in the RNAP/DNAP ratio, which increases from 2.2 to 5.8.

The failure of the DNAP to increase was confirmed in another test, the results of which are



Photomicrographs of a single explant of 12 day chick embryo heart at different stages of growth. A Sector of explant after 2 days in plasma with Tyrode solution as fluid phase. Embryo extract-serum mixture (EES) added at this time. B Same sector 24 hr later. EES renewed again at this time. C Same sector after further 24 hr. EES again renewed. D Same sector after further 48 hr. Note progressive thinning out of the central zone as growth proceeds. Magnification, $\times 52$.

shown in Fig 10 There is even a tendency for the DNAP to fall, particularly in the later stages when there was little change in the RNAP which reached its maximum at 96 hr The RNAP remained at this high level for a further 72 hr, during which period growth (as observed by the area of the culture) had apparently stopped However, the appearance of the cultures continued to alter, as a result of the thinning out of the central portions which became more diffuse and extensive

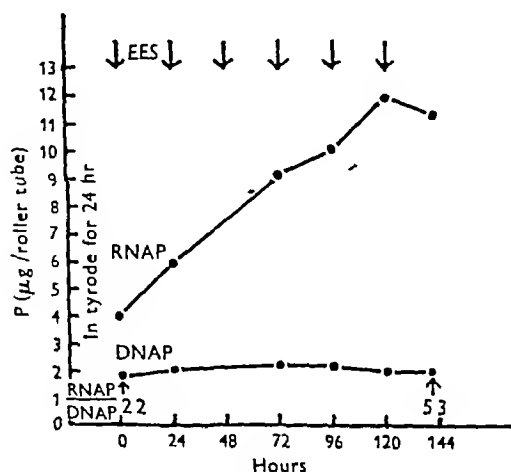


Fig 9 Changes in RNAP and DNAP in relatively large chick heart explants growing *in vitro* over 144 hr following addition and renewal of EES at intervals of 24 hr (as shown by arrows) See also collective legend for Figs 2-8

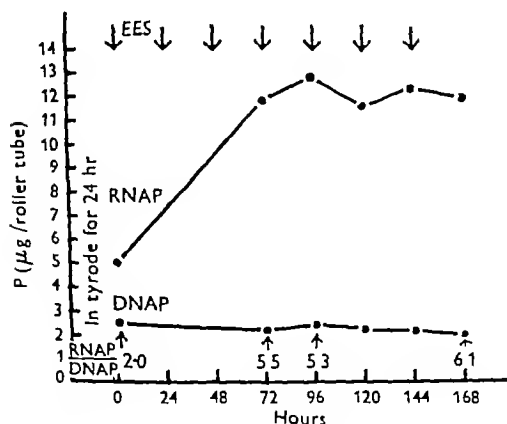


Fig 10 Changes in RNAP and DNAP in relatively large chick heart explants growing *in vitro* over 168 hr following the addition and renewal of EES at intervals of 24 hr (as shown by arrows) See also collective legend for Figs 2-8

In these two tests, as in the others already described, the explants were cut relatively large and each roller tube was calculated to contain 10-11 mg of fresh heart tissue at the start of the growth period Under such conditions it has been confirmed that the rise in RNAP is of the order of 250-300%, while the DNAP only rises in the early stages, at the most 25% above the initial resting values This rise

in DNAP is temporary and is followed after 96 hr by a fall in spite of the continued high RNAP values which are found during the same period

Effect of the size of cultures on their RNAP and DNAP changes during growth

A feature common to all the results so far described was our failure to obtain an appreciable and permanent increase in DNAP even in circumstances where growth of the cultures was apparently good There seemed to be some link between the thinning out of the central portions of the cultures, and the tendency of the DNAP to fall while this was occurring Such a fall in DNAP could be explained on the assumption that any increase in DNAP, by the production of new cells, was exceeded by its loss resulting from necrosis of the centre of the explants This would occur when the tissue pieces were large enough to prevent the cells in the centre from receiving an adequate supply of metabolites from the nutrient, or from getting rid of the waste products of their resting metabolism It was also possible that the amount of growth-promoting medium (0.5 ml was the maximum which could be used in our roller tubes) was insufficient to provide for the continued existence of the relatively large cultures which we employed

Support for these interpretations was available in the observations of Brues *et al* (1944) on the growth of cultures of minced chick embryo muscle in a peptone medium deficient in some of the factors thought to be necessary for growth They showed that an uptake of P from the medium could be used to measure growth in cultures of chick muscle, and found that, in a peptone medium insufficient for tissue synthesis, cultures continued to grow at the periphery while losing weight by necrosis of the central portions In tests using a medium fully adequate for growth, the cultures increased their P content until central necrosis balanced out or slightly exceeded growth at the periphery

Our technique was accordingly modified with the object of reducing the initial size and weight of the tissue cultures to a minimum This involved cutting the heart tissue into very small pieces, and planting them in rows of eight in 0.05 ml of plasma, instead of 0.1 ml as used previously The weight of tissue per tube in the resting state was estimated to be about 2 mg as compared with the 10-15 mg used in earlier tests This necessitated the pooling of material from two roller tubes in order to determine the amounts of RNAP and DNAP in the early stages of growth Once the cultures had grown appreciably it was possible to carry out determinations on the contents of single tubes In these tests, the RNAP was measured in amounts varying between 1.5 and 7.0 μg P and the DNAP, in amounts between 0.5 and 1.0 μg P All points are the mean of two determinations, and as shown in Tables 1 and 2 the final corrected RNAP and DNAP increases were at least twice as large as the corresponding plasma blank increases

The initial resting levels of RNAP and DNAP in the cultures themselves vary only slightly between 0.65-0.75 and 0.25-0.35 μg P per roller tube, respectively These

Table 1 *Comparison of changes in amount of ribonucleic acid phosphorus (RNAP) in tissue and plasma tubes*

Test	Plasma/tube (ml)	Hr in contact with embryo extract (EE) or embryo extract-serum mixture (EES)	RNAP at 0 hr (μ g)		RNAP increase at end of growth period (μ g)	
			Tissues*	Plasma	Tissues*	Plasma
40	0.3	48	6.43	0.45	+4.05	+0.43
41	0.3	11	4.65	0.26	+0.53	+0.21
42	0.3	36	5.71	0.26	+2.76	+0.71
46	0.3	24	3.12	0.40	+3.17	+0.02
49	0.3	58	3.32	0.16	+4.98	+0.56
50	0.3	120	3.98	0.12	+7.97	+0.48
51	0.3	168	4.94	0.10	+9.53	+1.29
53	0.3	48	5.74	0.12	+1.95	+0.39
57	0.3	49	2.64	0.12	+1.56	+0.01
Tests with smaller explants						
56	0.3†	120	1.28	0.18	+5.56	+0.50
58	0.3†	144	1.46	0.26	+5.20	+0.88
59	0.3†	144	1.18	—	+5.39	+1.58
62	0.3†	96 (EE)	1.55	0.04	+7.36	+3.28

* These are the figures obtained after correcting for plasma blank, and therefore the amounts represent the P gained or lost over and above that of the plasma blanks

† Plasma/2 tubes

Table 2 *Comparison of changes in amount of deoxyribonucleic acid phosphorus (DNAP) in tissue and plasma tubes*

Test	Plasma/tube (ml)	Hr in contact with embryo extract (EE) or embryo extract-serum mixture (EES)	DNAP at 0 hr (μ g)		DNAP increase at end of growth period (μ g)	
			Tissues*	Plasma	Tissues*	Plasma
40	0.3	48	2.41	0.19	+0.35	+0.04
41	0.3	11	1.85	0.06	-0.19	—
42	0.3	36	2.01	0.10	+0.04	-0.08
46	0.3	24	1.89	—	-0.12	+0.1
49	0.3	58	1.52	0.11	+0.24	+0.01
50	0.3	120	1.79	—	+0.22	+0.20
51	0.3	168	2.50	—	-0.68	+0.55
53	0.3	48	1.80	—	-0.28	+0.06
57	0.3	49	1.27	—	-0.28	+0.1
Tests with smaller explants						
56	0.3†	120	0.44	0.46	+0.88	-0.14
58	0.3†	144	0.60	0.20	+1.11	+0.36
59	0.3†	144	0.59	0.08	+0.67	+0.32
62	0.3†	96 (EE)	0.71	0.40	+1.1	+0.04

* See footnote, Table 1

† Plasma/2 tubes

cannot easily be further reduced since a lower limit is imposed on the size by the cutting and planting technique. With some practice it is possible to cut the tissues into pieces which have resting values close to 0.75 μ g RNAP and 0.35 μ g DNAP per roller tube. The same growth promoting medium (EES) was employed, and each roller tube again received 0.5 ml. renewed every 24 hr.

With this modified technique substantial increases in the amounts of both RNAP and DNAP have been obtained. In Fig. 11 the RNAP is seen to rise

steadily when determined at 48, 96 and 120 hr. On the other hand, there was a delay in the rise of the DNAP which showed little change at 48 hr over its initial value, though by this time the area of the cultures had slightly increased. Later, when the cultures were very extensive and the core apparently larger than at the resting stage, an increase in DNAP was recorded. At 96 hr the DNAP had risen by 80% and at 120 hr by 160%, the corresponding RNAP increases were 243 and 353%. The

ratio RNAP/DNAP, after rising to 7.4 at 48 hr, fell to 5.5 at 96 hr, and 5.2 at 120 hr, suggesting an approach to a new steady value. It also seems significant that this levelling out of the ratio occurs while the RNAP and DNAP are still increasing in amount.

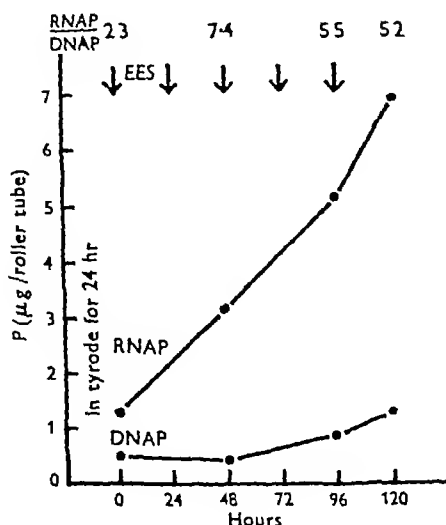


Fig 11 Changes in RNAP and DNAP in very small chick-heart explants growing *in vitro* over 120 hr following addition and renewal of EES at intervals of 24 hr (as shown by arrows). Results based on 48 pieces of tissue from two roller tubes (see also collective legend for Figs 2-8).

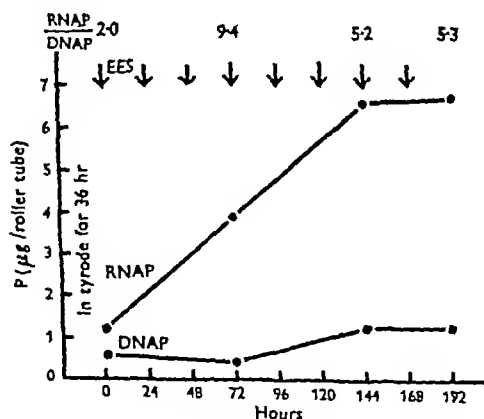


Fig 12 Changes in RNAP and DNAP in very small chick-heart explants growing *in vitro* over 192 hr following addition and renewal of EES at intervals of 24 hr (as shown by arrows). Results on the same basis as Fig 11.

Tests were run for longer periods in order to find the maximal values to which the RNAP and DNAP could rise under our experimental conditions. The results are shown in Fig 12. Both RNAP and DNAP contents were maximal at 144 hr after showing increases of 450% for the RNAP, and of about 100% for the DNAP, over the initial resting values. There was no change in the levels in the

subsequent 48 hr. The cultures did not appear to increase their area during this stage, but their centres appeared to spread out. This process was similar to the one occurring in the earlier tests, although there was now no tendency for the DNAP to fall at the same time.

Again in Fig 12 the rise in DNAP did not occur until after 72 hr and was, therefore, longer delayed as compared with the steady increase in RNAP obtained from at least as early as 48 hr. When maximal growth was reached the ratio RNAP/DNAP again levelled out at a value of 5.2. A final steady ratio of this order has been obtained in all the experiments in which substantial increases in both RNAP and DNAP were obtained. The other significant observation, which is a feature of all these tests, is the delay in the rise of DNAP for as long as 72 hr, even though the RNAP had increased appreciably in the same period.

Use of embryo extract (EE) as a growth-promoting medium

All the tests so far described have with one exception been carried out with EES as the growth-promoting medium. The preliminary tests, on which the decision to use this mixture had been based, had

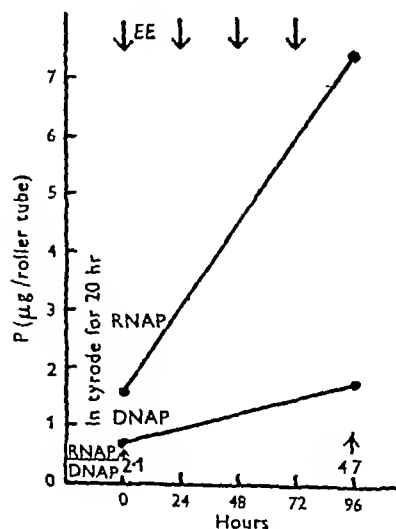


Fig 13 Effect of replacing EES by embryo extract alone in maintaining growth of very small chick-heart explants *in vitro*. The changes in RNAP and DNAP are followed over 96 hr, the EE being renewed every 24 hr (as shown by arrows). Results on the same basis as Fig 11.

never shown an increase in DNAP. They had only shown that in certain circumstances large increases in RNAP were obtained when the medium was EES. It was necessary, therefore, to repeat the comparison of the two media using the modified technique.

In two tests in which EE was compared with EES of closely similar N content, there was an indication that EE was just as good a growth promoter as EES, but these results were not considered conclusive. Other tests, however, have shown clearly that EE alone is fully adequate as a growth-promoting medium. Fairly concentrated extracts with an N content of about 80 mg/100 ml of extract have been used. As before, 0.5 ml was allocated to each roller tube, and the EE removed every 24 hr.

In one such test, the results of which are given in Fig. 13, good growth occurred over a period of 96 hr. This was seen to be accompanied by a 155% rise in DNAP and a 375% rise in RNAP, comparing favourably with the corresponding increases of 160 and 355% in a test in which EES was used (Fig. 11). It is concluded that EE alone as fluid phase is sufficient to supply all the necessary materials for growth of fibroblasts *in vitro*.

DISCUSSION

In analyzing the changes in RNAP and DNAP which occur in fresh explants growing *in vitro*, it is necessary to bear in mind that growth is not a simple process of multiplying cells, but a complex one in which the biosynthesis of cells at one point is accompanied by the breakdown of cells at another. Consequently any increases in either or both of the nucleic acids will be in proportion to new tissue synthesized in excess of tissue lost by the destruction of cells elsewhere.

In tests comparing the effects of embryo extract and Tyrode solution on the RNAP and DNAP content in the period immediately following planting, the fall in both, which leaves the RNAP/DNAP ratio unchanged, suggests that an intensive breakdown of damaged tissue is taking place. Within 20 hr the cultures in Tyrode solution have reached their resting level, and subsequently the RNAP and DNAP will only fall slightly if at all. Embryo extract reduces the fall, but does not prevent it occurring, and, although the cultures increase in area in this period, it is evident that the breakdown of cells must greatly exceed the synthesis of new tissue. In the second 20 hr, the position is apparently reversed in both sets of cultures for the RNAP increases appreciably, while the DNAP increases slightly or remains unaltered.

The growing of fresh explants *in vitro* also involves the selective multiplication of one of the cell types, in this case the fibroblast of the connective tissue, affecting principally the ratio of RNAP to DNAP. In recent years a number of workers (Davidson & Waymouth, 1944a, b, Schmidt & Thannhauser, 1945, Schneider, 1946) have shown that each tissue has its own particular content and ratio of RNA and DNA. These values will alter for any tissue according to its stage of development. For example,

there is a progressive fall in RNA, so that its concentration is appreciably lower in adult resting tissues than in developing tissues where a more intensive synthesis of protein is taking place (cf. Caspersson, 1947, Davidson & Waymouth, 1944a, b, Davidson & Leslie, 1948). Tissues, such as pancreas and liver, which are actively engaged in the synthesis of protein in the organism, also have RNAP/DNAP ratios considerably higher than the ratios found in other body tissues (Davidson, 1947). Thorell (1947a) has shown that the concentration of ribonucleic acid in haemopoietic cells falls from a value of 5% in the cytoplasm and nucleolar apparatus of the early cells to less than 0.5% in the mature cells. It would appear that this again involves a considerable drop in the RNA/DNA ratio from the exceptionally high values of the actively dividing cells.

For 12 day chick embryo hearts a RNAP/DNAP ratio of between 2.5 and 3.0 has been confirmed (Davidson & Leslie, 1948). In the tissue cultures at the time of planting the ratio lies between 2.2 and 2.5, which may indicate a fall in RNAP perhaps due to washing out of ribonucleoprotein by the saline in which the tissue is immersed for cutting. There are, however, no precise figures for the nucleic acid content of connective tissue, nor have we any information about the RNAP/DNAP ratio in the actively dividing cells, except that it is likely to be appreciably greater than in the corresponding resting cell. Interpretations of our results in growing cultures have, therefore, to take into account changed RNAP/DNAP ratios produced by the increasing predominance of the fibroblasts, some of which are actively synthesizing protein during the process of division.

In all the tests in which relatively large pieces of tissue were used as explants, any increases in DNAP are small in comparison to the RNAP increases, and of a temporary character (Figs. 2-10). When the DNAP does rise about 20% above its resting level it is usually after 48-72 hr of growth, in later stages, it shows a tendency to fall slightly. At the peak of the DNAP curve, the RNAP is usually double its resting value, and the cultures appear to have grown considerably. The RNAP continues to rise until 120 hr, and thereafter remains unchanged, even though the cultures alter in appearance and the DNAP shows a fall. Over this period the thinning out of the central portions, and the falling DNAP content in conjunction with the high RNAP, suggests that the loss of material is slightly greater than the gain from the continuing production of cells. The cultures have evidently reached the maximal size which can be sustained under these particular conditions of growth.

The final RNAP/DNAP ratio in all these tests with relatively large cultures lies between 5 and 6—a considerable increase over the resting ratio of

about 2.2. These high values must represent a mean for the cultures, since all the cells are not likely to be dividing and carrying out protein synthesis with its associated high RNAP content. Consequently cells in the process of division may have a higher value for the ratio and resting cells a much lower ratio.

After our failure in these tests to obtain a substantial rise in DNAP, the possibility remained that growth of explants from chick-embryo heart involved the replacement of the original cells by new ones with a higher RNAP and a much lower DNAP content. This process could quite well have contributed to the results we obtained.

The relative importance of these different processes has been largely settled by the tests in which visible growth was accompanied by substantial increases in both RNAP and DNAP. Clearly, reducing the initial size of the cultures produces the conditions under which an increase of DNAP can be recorded, and although this is about half the corresponding increase in the RNAP, it does not follow that the content of DNAP per nucleus is also half the original. It is likely to be closer to its original value in view of the exceptionally high RNAP concentrations found in actively dividing cells (Thorell, 1947*a, b*).

When suitably small explants are used, the RNAP rises five times above its initial value in 96 or 120 hr. This compares favourably with the two- to three-fold increase obtained in similar tests with larger cultures, and confirms the view that in the latter a loss of tissue at one point accompanies the increase of cells elsewhere. With the small pieces, once the maximum RNAP level has been reached, there is no tendency for it to fall.

Between 96 and 144 hr the DNAP has increased to twice or more than twice its initial content, and, like the RNAP, remains at this new level when growth apparently stops. This behaviour of both RNAP and DNAP suggests that the cultures grow to a limiting size, determined by the environmental needs of the cells. The outer limits of the cultures are set by the boundaries of the plasma clot, and their proximity to one another. The core of each culture will not increase in extent once further growth prevents the proper nutrition of the cells in the centre.

It seems highly significant that, in all the tests in which growth has been studied over long periods, the final RNAP/DNAP ratio lies between 5 and 6. For the tests with small pieces the ratio is less variable and lies between 5.0 and 5.5. This confirms the view that the tissue cultures develop to an equilibrium state in which new cells are being produced at the same rate as others are being destroyed. It can also be said that the ratio of RNAP/DNAP in actively dividing fibroblasts is at least 5, and very probably greater than 5. Since this ratio is obtained while both RNAP and DNAP are increasing, and still

below their maximal values (Fig. 11), there can be no further selective multiplication of cells in this active growth phase, as this would involve a changing ratio as the dominant type of cell increased in number.

Another feature of interest in all our tests is the observation that the RNAP invariably rises appreciably for some time before there is any significant increase in DNAP. There are two possible explanations for these results. One seems likely to apply to the tests of short duration, in which changes were followed at 2-hourly intervals (Figs 2-5). In these RNAP rises even when there is no visible sign of growth and no change, or a fall, in DNAP (e.g. Fig. 2). This behaviour strongly suggests that a synthesis of RNAP is occurring before additional DNAP is being produced by the appearance of new nuclei. As growth proceeds over longer periods another factor may come into play, it is possible that the new fibroblasts have a lower DNAP content than the average of 15 $\mu\text{g}/100\text{ mg}$ fresh wt found in the original tissue. As a result, the RNAP could increase appreciably for some time, while the DNAP remained unchanged or even showed a slight fall. Such a process could quite well be occurring in tests of the type shown in Figs 11 and 12. As late as 72 hr after the start of growth there is apparently no increase in DNAP, although by this time good growth at the periphery can be seen with the naked eye. Very probably the situation is complicated by the continuous breakdown of other cells, in which case the DNAP content of the new type of cell may be only slightly below that of the original cells. An actual increase in DNAP would, therefore, occur when the alteration of cell type in the cultures was nearly complete.

A study of the biogenesis of the nucleic acids, and the possible conversion of one into the other during growth, is complicated by the occurrence in most tissues of at least two relevant synthetic processes occurring simultaneously. There is the conversion of RNA into DNA, which has been postulated to account for the decrease in the initial store of RNA during the period in which the DNA is increasing in the developing echinoderm egg (Brachet, 1933, 1937). Such a process implies the earlier synthesis of the RNA from nucleotides or other smaller components. As Brachet (1945) has pointed out with reference to the chick embryo, this synthesis of RNA from its precursors masks the possible conversion of RNA into DNA in studies on the growing tissues. There is, of course, a third possible synthesis, that of DNA from components which are not derived from RNA (Brachet, 1947), and recent work of Schmidt, Hecht & Thannhauser (1948) suggests that this might possibly take place in the early development of the echinoderm egg. They found, on measuring the changes in RNA and DNA, that the RNA remained constant while the DNA increased as much as tenfold during the first 24 hr after fertilization. Their results do not, of course, exclude the possibility of conversion of RNA to DNA, but this could only occur if the turnover of RNA were shown to be very great during the rise

in DNA content. In phage infected bacteria, Cohen (1947) has shown that DNA is in fact synthesized in the absence of significant turnover of RNAP.

Our evidence of increased RNAP before there is any production of cells, or change in DNAP, supports the view that cells in the process of division synthesize RNA and protein first of all, the synthesis of DNA following later (Brachet, 1945, Painter, 1945). This does not necessarily conflict with the views of Caspersson and coworkers (e.g. Caspersson, 1947, Thorell, 1947*a, b*), who emphasize a different aspect of the process. Their evidence points to the nucleolus associated chromatin (containing DNA) as the initiator of the synthesis of ribose polynucleotides (or RNA) and the associated proteins abundant in diamino acids. These synthetic processes start at the nucleolus and spread outwards to the nuclear membrane, and to the cytoplasm. These results imply a primary synthesis of RNA by the cell as a means of increasing its protein content before its actual division occurs. There is as yet no clear indication that an increase in DNA comes first, although the evidence of Cohen (1947) and of Schmidt *et al.* (1948) mentioned earlier can be interpreted in the particular cases considered as favouring the independent synthesis of DNA from components not derived from RNA.

The fluctuations of RNAP only seem to occur in the period which immediately follows the addition of growth-promoting medium. It is also evident that this is the time during which RNAP is increasing most rapidly, for in the later part of the 24 hr period there is a definite fall in the rate at which it rises (Figs 3 and 6). This suggests that in response to the stimulus of fresh growth-promoting medium, there is an initial intense synthesis of RNA, which slows down prior to a rise in DNA (Figs 2, 4 and 6). However, it is difficult to account for the sharp fall in RNAP which was a regular occurrence and a feature of all the tests of this nature.

There are insufficient data on the changes in acid-soluble and lipid P to support any conclusions about their significance. The results of the test described earlier, however, suggest that the acid-soluble P in the extract and plasma is utilized during the period when increases in RNAP and lipid P are found in the growing tissues.

The experiments in which colchicine was added to the growth-promoting medium have clearly confirmed that we are measuring increases in both RNA and DNA as a result of their synthesis from materials present in the growth-promoting media. As there is a fall in both RNAP and DNAP with colchicine present, the increases with EES alone probably mask a simultaneous loss of material by the destruction of cells (Fig 8).

Although embryo extract-serum mixture (EES) has been used for most of the tests, there is no reason to believe that different results would have been found with embryo extract alone. Once substantial increases in both RNA and DNA are obtained by the use of very small cultures, both EE and EES are equally good in producing this effect.

SUMMARY

1 Changes in ribonucleic acid phosphorus (RNAP) and in deoxyribonucleic acid phosphorus (DNAP) have been followed in chick-heart fibroblast cultures growing in roller tubes *in vitro*, and a study made of the conditions under which increases in both components can be obtained. By renewing the fluid phase with embryo extract (EE) or embryo extract-serum mixture (EES) every 24 hr, it has been possible to run tests of varying duration up to 7 days.

2 When relatively large explants were used (fresh weight of 24 pieces of tissues estimated as 10–15 mg) only the RNAP content increased appreciably while visible growth was occurring. Over longer periods there was a relatively small and temporary increase in DNAP, although the RNAP continued to rise until it reached a steady level. In these tests the RNAP/DNAP ratio rose from initial values of 2.2–2.5 to final values between 5.0 and 6.0.

3 Appreciable and sustained increases in both RNAP and DNAP have been obtained when the cultures were grown from much smaller explants (fresh weight of 24 pieces of tissue estimated at 2 mg). Under these conditions the production of new cells evidently exceeded the loss of tissue arising from central necrosis of the cultures.

4 The final RNAP/DNAP ratio in all tests of long duration has been between 5 and 6. This marked rise during active growth supports the view that protein synthesis is accompanied by increased RNA concentrations in the cells.

5 In tests of short duration with relatively large explants, a rise in RNAP always preceded a rise in DNAP, and this is considered to indicate that RNAP is a precursor of DNAP in the dividing cells.

6 The delayed rise in DNAP, while visible growth is occurring in tests using small explants, is suggested as evidence of the replacement of the original cells by fibroblasts having a lower DNAP content.

7 In one test in which acid soluble P and lipid P were determined, the fall in the former in the tissue tubes suggests that this is incorporated in the nucleic acid P during the production of new tissue.

8 When colchicine was added to the extract, both the RNAP and DNAP fell in amount during the period when they would normally have increased or remained unchanged.

9 Embryo extract alone has been shown to be as adequate a medium for the growth of fibroblasts as a mixture of embryo extract and cockerel serum.

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The Irreversible Combination of Formaldehyde with Proteins

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The reaction of formaldehyde with proteins has been of interest for many years, owing to its industrial applications including tanning and plastics manufacture. Until recently the type of linkage formed was largely a subject for speculation rather than experiment. However, recent work has shed light on the details of the reaction. The literature has been adequately reviewed by French & Edsall (1945). Middlebrook & Phillips (1942, 1947) showed that at elevated temperatures the disulphide S of wool combines with formaldehyde, and that the reaction can be completely reversed by distillation with dilute phosphoric acid, it became apparent, however, that formaldehyde which combines with other groups cannot be completely removed in this way. Nitschmann, Hadorn & Lauener (1943) showed for casein that this method gave low results unless water was added, and a second distillation carried out, even so, the recovery of formaldehyde was not quantitative, and similar results were likewise obtained when 2N sulphuric acid was used instead of dilute phosphoric acid. When the distillation was continued to dryness, the results were unreliable, owing to the destruction of the protein, and the production of bisulphite-binding substances.

Wormell & Kaye (1945) and Fraenkel-Conrat, Cooper & Olcott (1945b) showed that the amide groups of proteins would combine with formaldehyde under acid conditions, and the latter authors found that the reaction was slow and greatly dependent upon formaldehyde concentration and reaction temperature. The present work was carried out to determine the effects of concentration of formaldehyde and pH on the amount of combined formaldehyde, which was stable to boiling dilute phosphoric acid and known as irreversibly combined formaldehyde.

METHODS

Materials

The wool used was a virgin Cape wool of about 64's quality, from which the tip ends had been removed, cleaned by successive extraction with ethanol, diethyl ether, and finally distilled water. Dark human hair was used for the supercontraction experiment. It was extracted by diethyl ether, any temporary set removed by allowing it to float freely in distilled water, and then carefully dried. Details of other protein preparations are as follows: casein, prepared from separated milk by precipitation with lactic acid (Van Slyke & Baker, 1918), N=15.7%, amide N=1.58%, collagen, sample from Dr D Jordan Lloyd, edestin, sample from Roche Products, Ltd, gelatin, Coignet 'Extra', amide N=0.45%, gliadin, sample from Prof A C Chibnall, glycinn, prepared from fat-free soya meal by the method of

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Jones & Moeller (1928), N = 17.47%, fully methylated wool, prepared by Dr H Lindley (see Blackburn, Carter & Phillips, 1941), haemoglobin (horse CO haemoglobin), prepared and heat-coagulated according to the method of Adair & Adair (1934), N = 17.2%, Fe = 0.33%, amide N = 1.08%, ovalbumin, sample of unknown origin, coagulated, dried and powdered. The proteins were extracted by diethyl ether, with the exception of the edestin and gelatin. The collagen was acetone extracted. They were all air dried, the moisture contents measured, and all determinations calculated on dry weight of protein.

Treatment and analytical methods

Formaldehyde treatment of hair Bundles of hair (1 g), tied by cotton thread, were boiled under reflux for 1 hr in 50 ml of 1% (w/v) formaldehyde solution, buffered in (i) borax adjusted with NaOH to pH 10.1 (final concentration of borax, 0.05M), (ii) NaH_2PO_4 adjusted with NaOH to pH 6.67 (final concentration of NaH_2PO_4 , 0.1M), (iii) sodium acetate adjusted with HCl to pH 1.00 (final concentration of sodium acetate 0.2M). The formalized hair was washed overnight in distilled water. Some of the hairs were boiled in 250 ml 0.1M H_3PO_4 until the volume of the latter was c. 10 ml, then washed and dried. No change of length of the hairs took place during any of these treatments.

Deamination Hair and other proteins were deaminated in a mixture of NaNO_2 and dilute acetic acid (Van Slyke's reagent), according to the technique of Speakman (1934).

Deamidation Proteins were partly deamidated by the following techniques: (i) in a solution of cetyl sulphuric acid and HCl (Steinhardt & Fugitt, 1942), (ii) in a solution of 1% NaOH for 40 hr at 45° (Wormell & Kaye, 1945), (iii) in a solution of NaNO_2 and acetic acid, containing 2N-HCl for 24 hr at room temp (Plummer, 1925; Fraenkel-Conrat, Cooper & Oleott, 1945a). This method would also deaminate the proteins, and remove the guanidino groups.

Estimation of tyrosine The intensity of the colour given by the action of α -nitroso β -naphthol on tyrosine was measured in the Spekker absorptiometer using green filters (Thomas, 1944). It was observed that cystine suppressed the colour. When the ratio of cystine to tyrosine was the same as that in wool, the value was lowered by 2.5%. In the case of wool, therefore, a correction factor was applied.

Bisulphite-stable fraction of the combined formaldehyde of formalized casein Casein (1.5 g) was boiled under reflux for 1 hr in 5 ml of pH 1 buffer and 5 ml of 8% (w/v) formaldehyde, 75 ml of 2.4% (w/v) NaHSO_3 solution were added, the mixture boiled under reflux for 20 min, left overnight, filtered, the precipitate washed, and the formaldehyde combined with the NaHSO_3 determined. The difference between the amount of formaldehyde taken and the amount in combination with the NaHSO_3 is, therefore, equal to the amount in combination with the casein and stable to boiling NaHSO_3 .

Estimation of irreversibly combined formaldehyde

Macro method Approx. 1 g of protein was weighed into a small round bottomed flask fitted with an air condenser having a ground glass joint, 5 ml of buffer (Na acetate + HCl, pH 0.46, 1.0, 2.0, 3.9, final concentration of acetate 0.4M) together with 5.0 ml of formaldehyde solution were added, the joint being lightly greased with petroleum jelly. The mixture was boiled under reflux for 1 hr, cooled, then quickly transferred, together with any washings, to a 250 ml

flask containing 100 ml of 0.3M H_3PO_4 . The liquid was then distilled, down to a volume of c. 5 ml, into a solution of NaHSO_3 (Nitschmann & Hadorn, 1941). Water was added to the flask and the distillation repeated, making a total of 4 distillations. Blanks containing known amounts of formaldehyde, without the addition of protein, were also distilled, and the amounts of formaldehyde distilled over were compared with direct estimations obtained by adding 5.0 ml of formaldehyde solution directly to the NaHSO_3 solution. A complete recovery of the formaldehyde was obtained. If the protein frothed unduly, anhydrous Na_2SO_4 (2 g) was added to the distillation flask. All the ground glass joints of the distillation apparatus were lightly greased. The solutions were left overnight, and the excess NaHSO_3 titrated with 0.1N I_2 using starch as indicator. Ethanol was then added to prevent oxidation, the NaHSO_3 formaldehyde compound decomposed by the addition of 1.0N Na_2CO_3 , and the liberated NaHSO_3 titrated with 0.1N I_2 (1 ml 0.1N $\text{I}_2 \equiv 0.0015$ g formaldehyde, Highberger & Retzsch, 1938).

Semimicro method When only small amounts of protein were available, the method was modified. The protein (c. 40 mg) was weighed in a glass tube sealed at one end, about 12 cm in length and 7 mm in internal diameter, 0.18 ml buffer and 0.18 ml formaldehyde solution were introduced by means of 2 Conway pipettes, which had been converted into burettes having capillary extensions with right angle bends attached to their tips, the flow of liquid being controlled by surface tension. The tube was immediately sealed in a blowpipe flame, then placed in an oven at 100° for 1 hr, and occasionally shaken. After this, it was broken in half and quickly dropped into 100 ml of 0.3N H_3PO_4 . The distillation was repeated as before. Blank distillation of known amounts of formaldehyde gave complete recovery.

Free amino nitrogen This was determined manometrically according to the technique of Peters & Van Slyke (1932), but in a blacked out chamber (Fraenkel-Conrat, 1943).

Free amino, guanidino, and part of the amide nitrogen The determinations were made in the Van Slyke microvolume apparatus (Plummer, 1917), in the presence of 2N HCl for 22 hr in a blacked out chamber (Plummer, 1925; Fraenkel-Conrat *et al.* 1945a).

Amide nitrogen Protein (0.25 g) was refluxed with 5N HCl (5 ml) for 4 hr, filtered, then diluted to 25 ml, 5 ml samples were made just alkaline to bromocresol green in the semimicro Kjeldahl apparatus. 5 ml of 2% borax solution were added to bring the pH between 8 and 9 (Lindley, 1946). NH_3 was distilled into a saturated solution of boric acid, and titrated with N/70 HCl, using a mixed indicator of bromocresol green and methyl red (Ma & Zuazaga, 1942).

Other methods Total nitrogen was determined by the micro Kjeldahl method (Chibnall, Rees, & Williams, 1943a) and cystine by the method of Shunohara (1935). Two dimensional paper chromatograms were made according to the technique of Consden, Gordon & Martin (1944), using water saturated collidine as one solvent, and water saturated phenol over 0.3% NH_3 solution in an atmosphere of coal gas as the other.

RESULTS

The effect of formaldehyde on the supercontraction of hair (Speakman, 1936) after boiling for 30 min in 5% (w/v) sodium bisulphite solution is shown in Table 1 (cf. Middlebrook & Phillips, 1942; Stoves, 1943).

Table 1 *Supercontraction of formaldehyde-treated hair, after boiling in 5% NaHSO₃ for 30 min*

pH of formaldehyde	Supercontraction		
	NaHSO ₃ only (a) (%)	Distilled in 0.1M-H ₃ PO ₄ before NaHSO ₃ (b) (%)	(b-a) (%)
10.1-9.4	3.3	14.0	10.7
6.7	19.8	32.3	12.5
1.0	5.0	28.6	23.6
Untreated	28.0	27.5	—
Deaminated			
Untreated	28.5	—	—
1.0	7.5	—	—
Deamidated (Steinhardt & Fugitt, 1942)			
Dissolved immediately			
Untreated	—	—	—
1.0	48.2	43.7*	—

* All the results are expressed as the mean of four determinations, except *, which is one determination only

In view of the instability to bisulphite of cross linkages between amino groups (Fraenkel-Conrat *et al.* 1945b), it is unlikely that these contribute to the diminished supercontraction shown by formaldehyde treated hair in boiling sodium bisulphite solution. This is confirmed by the fact that deaminated, as well as untreated fibres, which have been boiled in formaldehyde at pH 1, fail to supercontract on boiling in bisulphite solution (Stoves, 1944). On deamidation by the Steinhardt & Fugitt (1942) method (residual amide N, 0.4%), it is noticed that formaldehyde treatment confers no stability to supercontraction, although it does prevent the hair from being immediately dissolved in the boiling bisulphite solution. From these results, it is suggested that the values obtained in column 4 are due to cross linkages formed between the amide groups of glutamine and the guanidino groups of arginine (Wormell & Kaye, 1945, Fraenkel-Conrat *et al.* 1945b, 1946). From evidence to follow it is suggested that asparagine combines with formaldehyde, but it is unlikely to contribute to cross-linkage formation. The amount of formaldehyde in combination with casein which is stable to boiling bisulphite solution is equal to 3.67%, equivalent to 1.71% amide N, if one amide group combines with 1 mol of formaldehyde (actual amide N, 1.58%). When wool treated with formaldehyde at pH 1 was hydrolyzed and run on a two-dimensional chromatogram, no tyrosine spot was found (Middlebrook & Phillips, 1947). In order to ascertain if formaldehyde under these conditions does react with combined tyrosine, a sample of wool was treated with formaldehyde at pH 1, distilled in 0.3M-phosphoric acid, hydrolyzed for 4 hr in 5N-hydrochloric acid, and tyrosine determined by the method of Thomas (1944). The tyrosine content of untreated wool was 5.43%, and of treated 5.39%. It is unlikely, therefore, that formaldehyde has combined with tyrosine

under these conditions, but when liberated on hydrolysis it may be expected to combine with free tyrosine, histidine, etc (Neuberger, 1944).

In order to determine irreversibly combined formaldehyde, distillations were carried out in 2N-sulphuric acid until fumes appeared in the distillation flask. Confirming the observations of Nitschmann *et al.* (1943), more efficient removal of formaldehyde was obtained by repeated distillation with dilute phosphoric acid. Undue hydrolysis of protein was avoided under these conditions.

The effect of formaldehyde concentration on the amount irreversibly combined at pH 1 is illustrated in Fig 1. At concentrations above 4%, most of the proteins examined combine irreversibly with a small additional amount of formaldehyde. The effect of pH on the amount of irreversibly combined formaldehyde from a 4% (w/v) formaldehyde solution is illustrated in Fig 2. The values plotted in the two diagrams are the means of at least two determinations. The values of the minima of the curves in Fig 2, most of which occur between pH 1 and 2, are compared with the aspartic acid contents of the proteins, assuming that 1 mol of irreversibly combined formaldehyde is equivalent to 1 mol of aspartic acid. These values are summarized in Table 2, and compared with recent values for the aspartic acid content of the corresponding proteins. The increase in the amount of irreversibly combined formaldehyde at pH 3.9 is probably due to combination with histidine, as these values are roughly proportional to the histidine content, if 1 mol of formaldehyde combines with one histidine residue (cf. Neuberger, 1944). On increasing the concentration of formaldehyde above 4% (Fig 1), a considerable amount of histidine may react with formaldehyde at pH 1 (Neuberger, 1944), explaining the values obtained for carboxyhaemoglobin and casein, both of which have a high histidine content. When samples of these

latter proteins were hydrolyzed, after first being distilled in 0.3M-phosphoric acid to remove the reversibly combined formaldehyde, they were found to give no spot for histidine on a two dimensional chromatogram

cordingly deamidated by the Steinhardt & Fugitt (1942) technique. As these authors found it impossible to remove all the amide groups, those most easily removed were believed to arise from glutamine residues. It was also found that sufficient peptide hydrolysis had taken place to cause

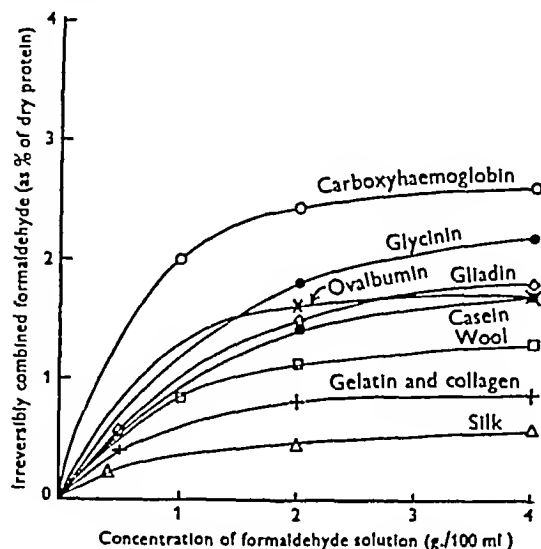


Fig 1 Effect of formaldehyde concentration on amount of irreversibly combined formaldehyde produced at pH 1 and 100° for 1 hr. Protein concentration 10 g/100 ml. Final concentration of sodium acetate in buffered solution 0.2N.

The increases at pH 0.46 are presumably due to partial hydrolysis of the proteins, followed by irreversible combination of formaldehyde with the products of hydrolysis.

The role of amide nitrogen in the production of irreversibly combined formaldehyde

If the formaldehyde combined irreversibly between pH 1 and 2 is combined with asparagine amide groups, deamidation should prevent this combination. Proteins were ac-

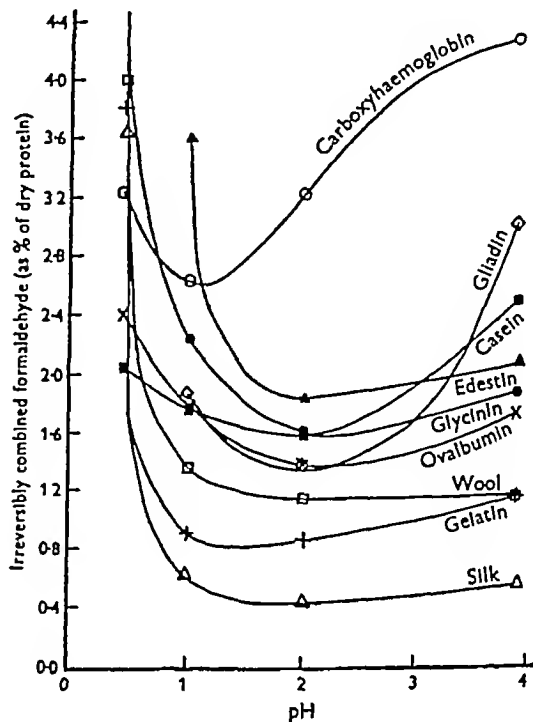


Fig 2 Effect of pH on amount of irreversibly combined formaldehyde produced in 4% (w/v) formaldehyde solution at 100° for 1 hr. Protein concentration 10 g/100 ml. Final concentration of sodium acetate in buffered solution, 0.2N.

an increase, rather than a decrease, in the amount of combined formaldehyde. Also, an increase in the time of treatment with the deamidating agent increased the amount of irreversibly combined formaldehyde without decreasing the amide N. Deamidation of wool in 1% NaOH solution

Table 2 Relation of irreversibly combined formaldehyde and the aspartic acid content of proteins

Protein	Irreversible formaldehyde* (%)	Aspartic acid calculated from treatment in		Aspartic acid from literature (%)		
		4% formaldehyde (%)	8% formaldehyde (%)			
Carboxyhaemoglobin	2.63	11.6	16.5	10.3 (4)	9.3 (1)	10.8 (6)
Edestin	1.84	8.2	12.0	10.2 (7)	12.0 (2)	
Casein	1.76	7.8	10.9	6.7 (1)	7.2 (6)	
Glycine	1.60	7.1	10.1		9.4 (7)	
Ovalbumin	1.38	6.1	6.8	9.3 (6)	8.1 (2)	6.2 (7)
Glutadin	1.35	5.9	—	0.8 (7)	1.4 (1)	3.3 (8)
Wool	1.17	5.2	5.2	5.7 (8)	7.3 (8)	
Gelatin	0.84	3.7	—	3.4 (3)	6.8 (2)	
Silk	0.44	2.0	2.9		2.8 (6)	

* Treatment in 4% formaldehyde

- (1) Bailey, Chibnall, Rees & Williams (1943)
- (2) Chibnall, Rees & Williams (1943b)
- (3) Dakin (1920)
- (4) Foster (1945)

- (5) Gordon, Martin & Synge (1941)
- (6) Hae & Snell (1945)
- (7) Jones & Moeller (1928)
- (8) Speakman & Townsend (1937)

(Wormell & Kaye, 1945) was unsatisfactory, as only a small amount of amide N was removed untreated wool, 1.15%, treated wool, 0.84%. Peptide hydrolysis took place, causing a higher value for irreversibly combined formaldehyde. In the case of wool, deamidation by the Plummer (1925) technique was only slightly more successful untreated wool, 1.15%, treated wool, 0.76%, untreated-wool cystine S, 3.25%, treated-wool cystine S, 0.53%. This treatment would also remove all the primary amino as well as the guanidino N.

In the case of carboxyhaemoglobin, no amide N was removed by the Plummer technique untreated protein, 1.08%, treated protein, 1.10%. The amount of irreversibly combined formaldehyde was approximately unchanged untreated protein, 2.62%, treated protein, 2.74%.

The amide N is somewhat higher than the value obtained by Rees (1946) after hydrolysis in conc. HCl at 37°. From the information he gives, it is unlikely that decomposition of serine and threonine can account for more than 0.03% N, as the hydrolysis is only carried out for 4 hr.

As shown by Sanger (1945), 2,4-dinitrophenyl derivatives of amino acids are remarkably stable to acid hydrolysis. A specimen of α -N-2,4-dinitrophenylasparagine was, therefore, prepared (Abderhalden & Blumberg, 1910), recrystallized and dried (m.p. 185°). A sample was treated with formaldehyde in aqueous acetone, containing a drop of conc. HCl, evaporated to dryness *in vacuo* several times with water, to remove excess formaldehyde, the residue distilled in 100 ml. 0.3M H_2PO_4 and the formaldehyde collected in $NaHSO_4$ solution. If 1 mol. of formaldehyde is equivalent to 1 mol. of 2,4-dinitrophenylasparagine, then the amount of formaldehyde found was equivalent to 118% of the 2,4-dinitrophenylasparagine taken.

Asparagine combines with formaldehyde to form 6-hydroxytetrahydropyrimidine-4-carboxylic acid (Schiff, 1900; Cherbuliez & Stavritsch, 1922), and in order to determine the stability of this compound a sample of asparagine was heated with a known excess of formaldehyde at pH 1 and 100° for 1 hr in a sealed glass tube. The tube was broken and the contents distilled in 0.3M H_2PO_4 . If 1 mol. of formaldehyde is equivalent to 1 mol. of asparagine, then the amount of combined formaldehyde, stable to distillation, was equivalent to 116.5% of the asparagine taken.

Table 3 The R_F values of asparagine and 6-hydroxytetrahydropyrimidine-4-carboxylic acid in phenol and *s*-collidine

Solvent	R_F values	
	Phenol 0.3% NH_3	<i>s</i> -collidine
Asparagine	0.4	0.2
6-Hydroxytetrahydropyrimidine-4-carboxylic acid	0.91	0.45

The R_F values (Consden *et al.* 1944) obtained on paper chromatograms are summarized in Table 3. Asparagine gave a brown, and 6-hydroxytetrahydropyrimidine-4-carboxylic acid gave a yellowish brown spot with ninhydrin, 6-hydroxytetrahydropyrimidine-4-carboxylic acid was partly stable to hydrolysis at the boiling point for 4 hr or at 38° for 22 days in 6N-hydrochloric acid. When this

hydrolyzed material was developed in phenol over ammonia, a purple spot of aspartic acid was obtained, together with a tailing yellowish brown spot of 6-hydroxytetrahydropyrimidine-4-carboxylic acid. When hydrolyzed in the presence of protein for 1 hr in boiling 6N-hydrochloric acid, the substance is completely decomposed to aspartic acid.

Table 4 Free amino N of proteins after reaction with nitrous acid

(Free amino N as percentage of total nitrogen, after reaction for 1 hr at 22° in 2N-HCl, all values are mean of two determinations)

	Blackened out chamber	In light
Carboxyhaemoglobin	5.55	6.59
Insulin (ash free)	5.65	5.88
Edestin	2.12	2.16

The effect of Plummer reagent on proteins

In order to determine the specific nature of the action of nitrous acid in 2N-hydrochloric acid on carboxyhaemoglobin, it was necessary to know the total free amino N of this protein, this was determined in the Van Slyke manometric apparatus, and also for insulin and edestin. The results are given in Table 4 which shows also the effect of blacking out the reaction chamber. A 40-W tungsten filament lamp was placed near the reaction chamber of the manometric apparatus when carrying out determinations in the light. The results obtained for carboxyhaemoglobin confirm the findings of Fraenkel-Conrat (1943) that unduly high values are obtained unless the chamber is blacked out. However, for insulin and edestin the effect is slight.

Fraenkel-Conrat *et al.* (1945a) stated that treatment of proteins with sodium nitrite and hydrochloric acid would yield nitrogen equivalent to their primary amino, amide and three quarters of their arginine, together with small amounts from unspecified sources. However, as it has already been shown, it was impossible to remove any of the amide N from carboxyhaemoglobin under these conditions. The value obtained as percentage of total protein N was 10.52%. The theoretical value for the sum of free amino N (5.55%) and guanidino N (calculated from arginine N as 7.2% of total protein N (Macpherson, 1946)), was 10.95%.

DISCUSSION

The action of formaldehyde on the disulphide S of wool has been studied in previous papers (Bowes & Pleass, 1939a, b; Middlebrook & Phillips, 1942, 1947). Wormell & Kaye (1945) showed that under acid conditions and in the presence of salts at 35° formaldehyde would combine with the amide groups

of proteins Fraenkel-Conrat *et al* (1945*b*) also showed that this reaction would take place at elevated temperatures and higher pH values. If hair fibres are first boiled in formaldehyde at pH 1, they become resistant to supercontraction in boiling bisulphite solution, and this also applies to hair fibres which have been deaminated before the formaldehyde treatment. On deamidation, however, the fibres supercontract, but, as has been shown, only a fraction of the amide groups are removed, and these probably consist of all the glutamine but none of the asparagine amide group. It is, therefore, concluded that the resistance to supercontraction is due to cross linkage formation between glutamine amide groups and others such as the guanidine groups of arginine (Fraenkel-Conrat & Olcott, 1946). These linkages are stable to boiling bisulphite solution, but can be completely removed by boiling the fibres in dilute phosphoric acid (see Table 1). The behaviour of fibres treated with formaldehyde at pH 10 can be explained by the formation of lanthionine cross linkages by the action of the alkaline buffer on the cystine cross linkages. As casein can combine with formaldehyde to produce linkages which are stable to boiling bisulphite solution, one formaldehyde mol being equivalent to one amide group, it is unlikely that cross linkages are produced between two amide groups. Although distillation in dilute phosphoric acid removes the formaldehyde cross linkages, some of the formaldehyde still remains in combination with the protein. It has not been found possible to remove this formaldehyde, and estimate it satisfactorily, except by difference. The amount of irreversibly combined formaldehyde tends to a maximum at 4% formaldehyde concentration (Fig. 1), and if the minimal values of irreversibly combined formaldehyde between pH 1 and 2 (Fig. 2) are compared with the aspartic acid contents of the proteins (Table 2), then 1 mol of formaldehyde is found to be equivalent to 1 mol of aspartic acid. From these results, it is suggested that all the aspartic acid is in the form of asparagine (cf. Damodaran, 1932; Chibnall, 1942), which combines with formaldehyde in acid, at elevated temperatures, to form 6-hydroxytetrahydropyrimidine-4-carboxylic acid residues, stable to acid hydrolysis (Cherbuliez & Stavritch, 1922; Levy & Silberman, 1937). A sample of the free acid was prepared, and found to be stable to distillation in dilute phosphoric acid. Although *N*-substituted asparagine would combine with formaldehyde, ring closure apparently did not

occur, for this material was completely decomposed on distillation in dilute phosphoric acid.

Fully methylated wool combined irreversibly with less formaldehyde at pH 1 than untreated wool. This may be due to methylation of the asparagine amide groups.

Deamidation by the Plimmer (1925) technique removed only a part of the amide nitrogen and the amount of irreversibly combined formaldehyde was approximately unchanged. Of the proteins which were deamidated by this technique, the residual amide was approximately equivalent to the aspartic acid content. It is, therefore, probable that deamidation by the Plimmer technique only removes the glutamine amide nitrogen from proteins. It is significant that no deamidation took place in the case of carboxyhaemoglobin, where, if the aspartic acid content is taken to be 10.3% (Foster, 1945), all the amide nitrogen could be in the form of asparagine. Fraenkel-Conrat *et al* (1945*b*) refer to the difficulty of removing all the amide nitrogen, and their results could be explained in this way. If confirmed, this might be used as a method to estimate the distribution of amide nitrogen in proteins between glutamine and asparagine. It should be noted that in all the proteins examined there is sufficient amide nitrogen to account for all the aspartic acid as asparagine. The free carboxyl groups in these proteins may arise from the glutamic acid residues and terminal amino acids.

SUMMARY

1 In acid conditions at 100° proteins combine with formaldehyde, some of which cannot be removed by distillation in acid.

2 In the proteins examined, probably all the aspartic acid is in the form of asparagine residues, and the glutamic acid is in the form of glutamine and glutamic acid residues.

3 Formaldehyde forms cross linkages between the glutamic amide groups and other groups, possibly the guanidine groups of arginine. These cross linkages can be broken down by distillation in dilute phosphoric acid.

4 Formaldehyde probably combines with the asparagine amide groups to form 6-hydroxytetrahydropyrimidine-4-carboxylic acid, which is stable to boiling dilute phosphoric acid.

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Studies on the Fermentation of Ceylon Tea

8 FURTHER OBSERVATIONS ON THE RELATIONSHIP OF TEA FERMENTATION TO NORMAL RESPIRATION

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The original hypothesis of Roberts that all normal respiration ceases when tea leaf is rolled in the process of manufacture, and that the carbon dioxide observed during fermentation is due to a secondary carbohydrate oxidation by the o-quinones, has been further amplified in his two later communications (Roberts, 1941, 1943) This hypothesis, however, is in conflict with the finding in this laboratory

(Sreerangachar, 1941) that when the leaf is mechanically damaged, as in tea rolling, a certain amount of normal respiration still persists, thus accounting for the carbon dioxide produced by the fermenting leaf This difference of opinion makes a critical examination of the evidence presented on both sides desirable, especially since these investigations, seriously interrupted during the war, can now be continued

The problem centres round two main issues First, is there any residual normal respiration in the

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leaf which has suffered damage similar to that in tea rolling, and, secondly, do the tannin *o*-quinones play a role in the carbohydrate oxidation in fermenting leaf? The purpose of the present paper is to discuss the evidence relating to these two issues in the light of some fresh results obtained here

EXPERIMENTAL AND RESULTS

Residual respiration in factory rolled tea leaf

Residual respiration has been previously established in fresh or withered tea leaf after mincing but not in factory-rolled leaf. Whether or not respiration is completely suppressed in the rolled leaf depends on the nature and extent of the mechanical injury inflicted on the leaf tissues in rolling. The breaking up of leaf depends upon many factors such as its physical condition, the mechanics of rolling and so on, but even after the hardest rolling now in practice one can always pick out several green and undamaged pieces of tissue from a mass of rolled leaf. Complete rupture of all the cells does, no doubt, result in total suppression of respiration, but such extreme damage is never accomplished in a tea roller in Ceylon.

The most convincing proof of the presence of respiring tissue in rolled leaf is now furnished by direct experimentation. Measurements of respiration in this and subsequent experiments were done by conventional manometric methods using the Barcroft differential apparatus. Withered leaf, after one or more rolls of 30 min in the factory, was

well washed with water to remove the expressed polyphenols, both oxidized and unoxidized, which alone could provide material for Roberts's mechanism, 200 mg of this tissue were suspended in 2 ml of water and the respiratory CO_2 measured at 28° .

Table 1 gives results typical of those obtained in several experiments. Different leaf has been used in the different experiments shown in the table.

Polyphenol oxidation during tea fermentation

The fermentation period in the factory varies generally from 2.5 to 4 hr, and it is almost certain that the polyphenolic constituents are not completely oxidized within that period, as is shown by a typical case (Table 2).

Exp 1 was carried out on factory samples whilst for exp 2 withered leaf was minced in the laboratory. The total oxidizable matter, which was determined by the method of Shaw (1935), is proportional to the amount of polyphenols present (Eden, 1935), the extent of oxidation being indicated by the fall in total oxidizable matter. Part of this fall is probably due to condensation phenomena, but the fact that there is a correspondence between the fall in total oxidizable matter on the one hand, and the O_2 uptake (free from condensation) on the other, shows that the main conclusions are not much affected by such condensation changes. The O_2 uptake was determined by means of a macro-absorption apparatus using 20 g of withered leaf. The degree of accuracy obtained with this apparatus is not as high as in the

Table 1 *Residual respiration in washed rolled leaf*

Time (min)			15	30	60	90	120	150	180	210	240
CO_2 production (μl)	Exp 1	Withered leaf, 1 roll	11	20	47	60	75	86	98	102	111
	Exp 2	Withered leaf, 3 rolls	14	22	35	44	54	60	63	—	—
Time (min)			5	10	15	30	45	60	75	90	
CO_2 production (μl)	Exp 3	Withered leaf rolled 1st dhool*	9	16	21	32	44	55	69	76	
		4th dhool	0	5	5	7	12	18	23	28	
		Withered leaf rolled in Clivemeare† roller	2	5	9	12	14	18	21	25	

* Rolled leaf sifted through a standard mesh is called a dhool. 4th dhool has had longer rolling than the 1st dhool.

† Clivemeare roller is a new type of roller which very quickly reduces green leaf almost to a pulp. It has not yet been adapted for commercial manufacture of black tea.

Table 2 *Oxidation of tea polyphenols during fermentation*

Period of fermentation (hr)	Total oxidizable matter in terms of ml 0.05N-thiosulphate/g dry matter		O_2 absorption during fermentation in exp 2 (ml/g dry wt)
	Exp 1	Exp 2	
0	—	247	—
0.5	273	—	—
1	—	223	3.76
2	258	204	5.71
4	208	196	7.87
6	196	184	9.18
24	171	147	11.03

usual manometric experiments, but the results have a special value in that the conditions of leaf fermentation in this apparatus closely approximated to those in the factory

It will be seen that at the end of 4 hr fermentation, which is perhaps the maximum under factory conditions, the amount of oxidation is only 60–65 % of that attained after 24 hr. It is also clear that oxidation of polyphenols continues for periods much longer than the time usually allowed for fermentation in the factory

Inhibition of respiration by tea-tannin oxidation products

In tea fermentation Roberts has observed that, after completion of tannin oxidation, CO_2 production falls to a very low level, and has, therefore, assumed a close dependence of the latter on the former. Evans (1928), however, found that the CO_2 production decreased only slightly from the first to the seventh hour of fermentation. My own results (Sreerangachar, 1941) have shown a slight fall in the CO_2 output towards the end of fermentation, but this is perhaps associated with the inhibitory effect of oxidized polyphenols on the normal respiration. Richter (1934) has shown that catechol oxidase is inhibited by o-quinone, and Boswell & Whiting (1938) have established the inhibitory nature of the quinonoid compounds on the normal respiratory system of potato. Other instances of oxidative quinone inhibitions are mentioned by Lipmann (1943). The possibility, therefore, exists that in tea fermentation a similar inhibition of respiration may occur.

In order to test this, the respiration of fresh leaf was measured in the presence of oxidized tea polyphenols and compared with the values obtained free from this influence. The figures in Table 3 represent the respiratory CO_2 of 200 mg of fresh leaf suspended in 2 ml of water in one case, and 2 ml of black tea infusion (2.82 g/150 ml water) in the other. The leaf samples were derived from the second leaf of a normal flush containing a bud and two leaves. The flushes employed in any one experiment were from the same bush. The results were confirmed by several similar experiments, they demonstrate that there is an inhibition of the normal respiration of the leaf by the tea-tannin oxidation products, and explain why there is a fall in respiratory rate towards the end of tea fermentation.

Stimulation of respiration by catechol

Boswell & Whiting (1938) have conclusively shown that on adding catechol to respiring potato slices both the O_2 uptake and the CO_2 output are increased. It must be emphasized that this stimulation of CO_2 output is not due to an *in vitro* reaction in which the o-quinone acts as H acceptor in carbohydrate oxidation, but that it is due to a stimulation of the *in vivo* respiratory system. Similarly, when catechol is added to the fermenting leaf-mince system, in which there is a certain amount of residual respiration, stimulation of the latter could naturally follow.

Results given in Fig. 1 afford confirmation of this hypothesis in that there is a stimulation of tea-leaf respiration by catechol. This has been investigated

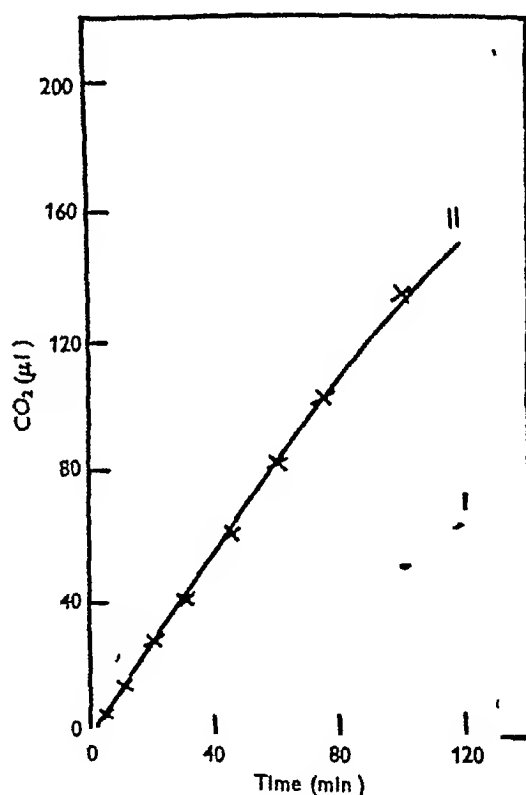


Fig. 1 Respiration of tea leaf I, in water, II, in presence of 0.05M-catechol

by suspending tea leaf in 2 ml of 0.05M-solution of catechol and taking measurements in a Barcroft respirometer. To facilitate observation of results from the moment of polyphenol addition, catechol (0.5 ml, 0.2M) was held in a dangling tube which was

Table 3 *Inhibition of respiration by oxidized tea polyphenols*

Time (min)	15	30	45	60	75	90	105	120
Leaf suspended in water ($\mu\text{l CO}_2$)	23	42	63	86	109	128	151	174
Leaf suspended in tea infusion ($\mu\text{l CO}_2$)	16	30	46	62	79	95	104	120

dislodged and mixed with the contents of the flask (1.5 ml of water) at zero time. As before, the leaf samples were derived from the second leaf of the flushes from the same bush. It is clear that the stimulation of CO_2 output, observed by Roberts when catechol was added to fermenting tea leaf, is due more to the stimulation of normal respiration in undamaged portions of the leaf than to a greater production of *o*-quinones causing a higher carbohydrate oxidation in the damaged tissue. Any possible interference in the above experiment by reactions at the cut ends of the leaf was eliminated by a prior washing of the cut pieces, and it was found by a control experiment that when the tissues were so washed direct oxidation of added catechol was negligible.

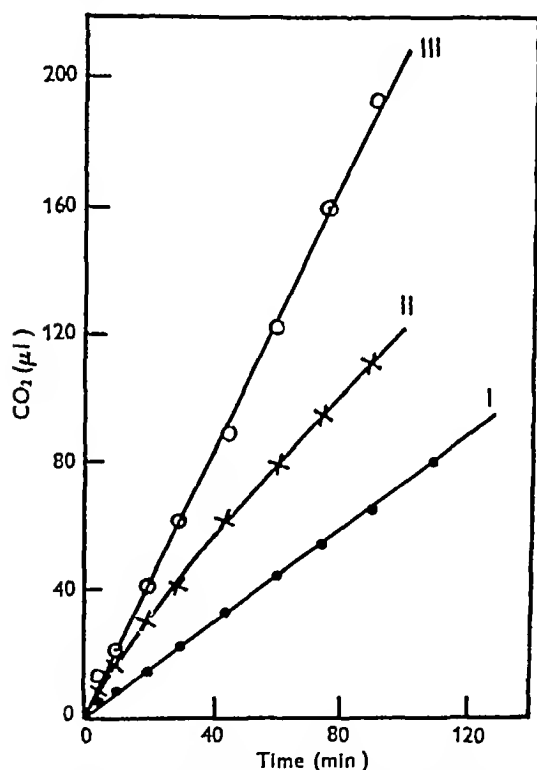


Fig. 2. Respiration of tea leaf I, normal, II, in presence of 0.05M catechol, leaf cut into 5 pieces, III, as in II, but leaf cut into 28 pieces.

There is reason to believe, however, that the amount of the cut edges influences this stimulation of respiration by catechol. Thus when 200 mg of leaf were cut into twenty-eight pieces the stimulation was higher than when the same amount of leaf was cut into five pieces (Fig. 2). It appears, therefore, reasonable to expect that, in the case of finely chopped tissues or of leaf mince such as that used by Roberts, the stimulatory effect would be still higher than that observed here. The exact mechanism of this stimulation calls for further study but the observation provides perhaps a more suitable explanation of Roberts's results.

DISCUSSION

By using an attachment which would grind nuts to a paste Roberts claims to have caused more extensive structural damage than I did, and he holds that factory rolling causes similar damage to the leaf. In his opinion the damage caused in both cases is, as already mentioned, sufficient to suppress completely all normal respiration. It can be shown, however, that even under these conditions of grinding there exists an appreciable amount of normal respiration in the finely minced tissues. Roberts & Sarma (1940) have given Q_{O_2} and RQ values for several plant tissues ground by Roberts's method. Let us first consider the two simple systems, e.g. the two non-tanniferous tissues *Tropaeolum majus* and *Hibiscus rosasinensis*. Calculated on the Q_{O_2} and the RQ figures these tissues will have a carbon dioxide output of 1.98 and 1.60 $\mu\text{l}/\text{mg}$ dry wt of tissue/hr respectively. These values are of the same order as that found by Jensen (1923) for *Tropaeolum majus* (1.29). As these tissues are non-tanniferous there is no question of polyphenol oxidation occurring after mincing, and since the RQ is very near unity the carbon dioxide is presumably due entirely to normal respiration of undamaged tissue.

Only four out of the 15 plant tissues for which Roberts & Sarma have given the RQ values seem to have carbon dioxide outputs outside the above range. It is quite possible that an explanation of their higher carbon dioxide outputs can be found on the basis of the wide variation of respiratory intensities in different plant species.

Roberts & Sarma (1940) place undue reliance on the correlation between tannin content and Q_{O_2} . This correlation $r=0.6160$ is vulnerable at two points. The mere establishment of statistical significance is no guarantee of an important degree of common causation between the variables. Translated into terms of the predictability index I (Trelear, 1936) the value is 0.2122, from which it is apparent that only 21% predictability of the dependent variable is possible. Further, even if a high and significant value of r were shown, this would not establish the causal sequence between the *o*-quinone production and carbohydrate oxidation, for at no point do Roberts & Sarma differentiate between oxygen used for carbon dioxide production and oxygen used to produce *o*-quinone destined to form condensation products. This latter oxygen being the greater part (*vide* the low values of RQ) is undoubtedly capable of producing a correlation that will effectively mask any correlation between tannin and oxygen used for carbohydrate oxidation. Without the establishment of a partial correlation which cannot be calculated from the data, the existing

correlation is likely to be spurious as a basis for stipulating common causation

The amount of carbon dioxide produced in the various tissues is a better indication of the role of tannins as H carriers in carbohydrate oxidation than the Q_{O_2} values. There appears, however, to be no correlation between the carbon dioxide output and the tannin content, as the correlation coefficient obtained ($r=0.29$) falls much short of significance (theoretical value = 0.5139 for $P=0.05$). If the normal respiration has not completely ceased on grinding the non-tanniferous tissues, there is no reason why we should assume anything different in the case of tea leaf.

Perhaps the most important evidence which is considered to support Roberts's theory of the role of o-quinones in carbohydrate oxidation is the stimulation of the carbon dioxide output in the fermenting leaf by added polyphenols (catechol). It would indeed be difficult, as Roberts states, to explain this stimulation on my hypothesis, were it not that polyphenols like catechol are found to have a stimulating effect on the normal respiration of leaf (Fig. 1).

Roberts's (1941) picture of the equilibrium of the oxidation-reduction processes within the intact plant cell may be essentially correct, and it may also be possible that mechanical damage destroys the organization of the reactive surface, resulting in the cessation of normal respiration and anaerobic fermentation. The question, however, still remains as to whether all the active respiratory surface is completely disorganized by such mechanical injury as that occurring in a tea roller. As mentioned earlier, I have not reproduced exactly the conditions of the Tocklai experiments, nor is it possible to do so in the absence of detailed information about the grinding which is at present lacking, but this does not detract from the validity of my conclusion, especially since it can be shown from Roberts's own data that

respiration was not completely suppressed in his experiments as well.

It has been made clear (Sreerangachar, 1943a) that the cytochrome theory of tea fermentation cannot be accepted in view of the spectroscopic and manometric findings. The nature of the enzyme responsible for fermentation has been further investigated, and it has been shown (Sreerangachar, 1943b) that tea polyphenol oxidase is also a copper-protein compound. The evidence presented here against Roberts's hypothesis of the relation between normal respiration and fermentation weakens yet another link in the mechanism of tea fermentation advanced by him.

SUMMARY

1 Residual respiration has been experimentally demonstrated in the factory-rolled withered tea leaf, thus establishing that normal respiration is not totally suppressed by the mechanical damage inflicted in rolling. Also the existence of residual respiration is inferred from Roberts's (1941, 1943) own data.

2 The main items of Roberts's evidence have been discussed and other explanations furnished for them in the light of the above observation.

3 Tea-tannin oxidation products produced during fermentation inhibit the normal respiration of the leaf, but addition of fresh polyphenols such as catechol has a stimulating effect on respiration. These observations explain the so-called dependence of carbohydrate oxidation on the tea-tannin o-quinones.

The author wishes to thank Mr J. Lamb, Biochemist, for many helpful suggestions and advice, and Dr R. V. Norris, Director, for his continued interest in this investigation. Thanks are also due to Dr T. Eden, Agricultural Chemist, for his critical reading of the manuscript.

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The κ -Toxin (Collagenase) of *Clostridium welchii*

2 THE EFFECT OF ALKALINE pH AND HEAT

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In a previous paper (Bidwell & van Heyningen, 1948) the partial purification and characterization of the κ -toxin (collagenase) of *Clostridium welchii* was described. It was observed that dialysis of purified enzyme preparations against borate sodium carbonate buffer at pH 10.2 resulted in an increase in the activity of the enzyme against 'azocoll' (commercial hide powder to which an azo dye has been coupled, see Oakley, Warrack & van Heyningen, 1946), and a marked decrease in the immunological test dose using azocoll as indicator. The effect of dialysis at pH 10.2 has since been repeatedly observed. In six cases there was a considerable rise in enzyme activity ('recovery' 120–210%), in four cases the activity was practically unchanged (90–110%) and in four cases there was considerable loss. In all cases we confirmed that there was a marked decrease in the immunological test dose with azocoll as indicator, but the extent of this apparent increase in combining power varied considerably ('recovery' 145–275%, mean value 188%).

It was found by Dr A. Pirie (private communication) that one of our collagenase preparations was active in disintegrating ox cornea collagen, but that a later sample of 'collagenase', which we expected from our tests on azocoll to be about as active as the first, was totally devoid of the power to digest ox cornea collagen. Reference to our records showed that this second sample had, at an earlier stage in its history, been dialyzed at pH 10.2. Several samples which had been thus treated were tested for ability to disintegrate muscle and collagen 'paper' (Oakley *et al.* 1946), and in every case it was found that although the material was still highly active in bringing about solution of azocoll, no trace of activity against muscle or collagen paper remained. Further investigations on the effect of alkaline pH on collagenase preparations are reported in the present paper.

METHODS

The activity of enzyme preparations in bringing about solution of azocoll was measured in Q units/ml (Bidwell & van Heyningen, 1948). It must be understood that, as the work to be described will demonstrate, such enzymic activity could be due to true collagenase (i.e. unaltered collagen as specific substrate) and other enzymes which can attack azocoll but not collagen. In the sequel the term

'azocoll enzyme' will be used to denote enzymic activity measured against azocoll. Collagenase antigen was measured by its combining power in κ units/ml by the methods of Oakley *et al.* (1946) using serum R 8537 (180 anti κ units/ml) as standard with (a) azocoll, (b) collagen paper, and (c) muscle as indicators, leucithinase antigen in Le units by the method of van Heyningen (1948), total nitrogen by the micro Kjeldahl technique, pH values were measured electrometrically at 15° using a glass electrode. For the composition of the borate buffers used see Clark (1928).

EXPERIMENTAL AND RESULTS

Four separate batches of *Cl. welchii* type A crude filtrate were worked up according to the method of Bidwell & van Heyningen (1948). Table 1 shows the stages in the purification process and a comparison at each stage between the azocoll enzyme content and the 'collagenase' antigen content as determined with the three indicators azocoll, collagen paper and muscle for a typical purification. It is probable that the first ammonium sulphate precipitation results in a change resembling toxoiding in that the enzyme activity appears to be diminished in relation to total antibody-combining power, but active enzyme is recovered preferentially to total antigen in the calcium phosphate treatment. It is clear from Table 1 that true collagenase antigen, measured using muscle or collagen paper as indicator, is retained throughout the stages of the purification.

The effect of alkaline pH on collagenase preparations

Preliminary work was done entirely on purified enzyme preparations by dialyzing against borate Na_2CO_3 buffer at pH 10.2. It should be emphasized that in all cases the pH was restored to c. 7.4 before testing for enzyme or antigen content.

The effect on purified enzyme preparations of (a) dialyzing against borate buffers of varying pH and (b) adjusting the pH by alkali. The enzyme preparation used (B 476 G) had been purified by the method of Bidwell & van Heyningen (1948). It contained 1100 Q units of collagenase/ml, 3900 κ units of collagenase antigen/ml (using azocoll as indicator) and 1.78 mg non-dialyzable nitrogen/ml. For the following experiments it was diluted 1/50 in borate buffer pH 7.4. (a) Samples were dialyzed at c. 2° against seven daily changes of borate buffers as follows: Palitzsch's borate buffer pH 7.35 and 8.47, 0.05M sodium borate pH 9.17 and Kolthoff's borate Na_2CO_3 buffers pH 9.59, 10.34 and 10.73, respectively, and each was then redialyzed to equilibrium against borate

Table 1 *Relation between activity of collagenase enzyme (measured in Q units/ml) and apparent collagenase antigen content, using as indicator (a) azocoll (κ_A units/ml), (b) collagen paper (κ_O units/ml) and (c) muscle (κ_M units/ml)*

(c) muscle (κ_M units/ml)		Relative activities						
Stage of purification	Q units/ ml	κ test doses/ml						
		κ_A units/ ml	κ_O units/ ml	κ_M units/ ml	κ_A units/ ml	κ_A units/ ml	κ_A units/ ml	κ_M units/ ml
Crude filtrate	1 67	4 2	3 9	3 1	1 1	1 4	2 5	1 8
Precipitated with $(\text{NH}_4)_2\text{SO}_4$ (60 kg/100 l), scum dissolved in water	48	200	125	180	1 6	1 1	4 2	3 7
Adsorbed on calcium phosphate and eluted with 10% (w/v) $(\text{NH}_4)_2\text{SO}_4$ (eluate I)	19	49	31	31	1 6	1 6	2 6	1 6
Eluate I dialyzed against tap water	13 7	33	22	22	1 5	1 5	2 4	1 6
Second calcium phosphate treat- ment, giving eluate II	28 4	76	48	51	1 6	1 5	2 7	1 8
Decolorized with 1% (w/v) charcoal	32	67	40	46	1 7	1 5	2 1	1 4
Dialyzed against tap water	22	50	35	35	1 4	1 4	2 3	1 6
Solution of precipitate obtained on adding 2 vol saturated $(\text{NH}_4)_2\text{SO}_4$ solution	466	1160	500	670	2 3	1 7	2 5	1 3
Dialyzed against borate buffer pH 7 4	355	1240	510	480	2 4	2 6	3 5	1 4

buffer pH 7 4. Volume changes were negligible (<2%). The possible specific effect of the carbonate ion introduced at the change from Palitzsch to Kolthoff buffers was considered, but, in a similar experiment the effect of dialysis against a carbonate bicarbonate buffer of pH 9 1 (Delory & King, 1945) was shown to be the same as when 0 05M-sodium borate at the same pH was used. (b) A second set of samples of diluted enzyme preparation were adjusted with 0 1N-NaOH to pH values corresponding to those of the buffers used in the dialysis experiment. Each sample was divided into three equal parts which were then treated separately as follows: (1) Heated at 37° for 0 5 hr, cooled to 15° and pH readjusted to 7 4. (2) Left overnight at c 2°, pH readjusted to 7 4. (3) Left 7 days at 2° and pH readjusted to 7 4.

The preparations were tested for activity against azocoll (in Q units/ml) and for κ antigen content using both azocoll and muscle as indicators. The results are expressed in Figs 1, 2 and 3 as a percentage of the value for the preparation which had remained at pH 7 4 throughout the experiment. In the concentration used the enzyme appeared to be stable at pH 7 4 in all the experiments.

Where the enzyme was kept throughout in the cold, the activity against azocoll was minimal after treatment at pH 8 5–9 0, with increasing activity after treatments at pH 9–10 3 and then a very sharp drop after being held at pH 10 8 (Fig. 1). True collagenase antigen (tested against muscle, see Fig. 2) was appreciably unstable at pH 9 0 and no muscle disintegrating activity remained after treatment at pH 10 3, since even in the total absence of antiserum the undiluted enzyme preparations were

without effect on muscle or collagen paper. The results were qualitatively the same whether the pH was changed by simple adjustment or by dialysis, they cannot, therefore, be ascribed to the removal by dialysis of some factor which dissociates from the enzyme at these alkaline pH values, but may be due to the formation from some inactive precursor of a second enzyme, which can disintegrate azocoll but not true collagen.

The effect on the apparent combining power as tested using azocoll as indicator is shown in Fig. 3.

When crude enzyme preparations were dialyzed against borate- Na_2CO_3 buffer at pH 10 2 the results were qualitatively similar to those found for purified enzyme preparations and so will not be given in detail. In all cases most of the leucithinase activity appeared to be destroyed.

The effect of heat on purified collagenase preparations. Equal volumes of a partially purified collagenase preparation diluted in borate buffer pH 7 4 were heated in a water bath at various temperatures for 10 min and then immediately cooled. Determinations of azocoll-enzyme activity and combining power with muscle and azocoll as indicators were made. Fig. 4 shows that loss of muscle disintegrating activity was apparent after heating to 50°, and complete after heating to 56°, but there was a slight rise in azocoll-disintegrating activity after heating to 50°, and at least 50% of the azocoll-enzyme activity remained after heating to 56°.

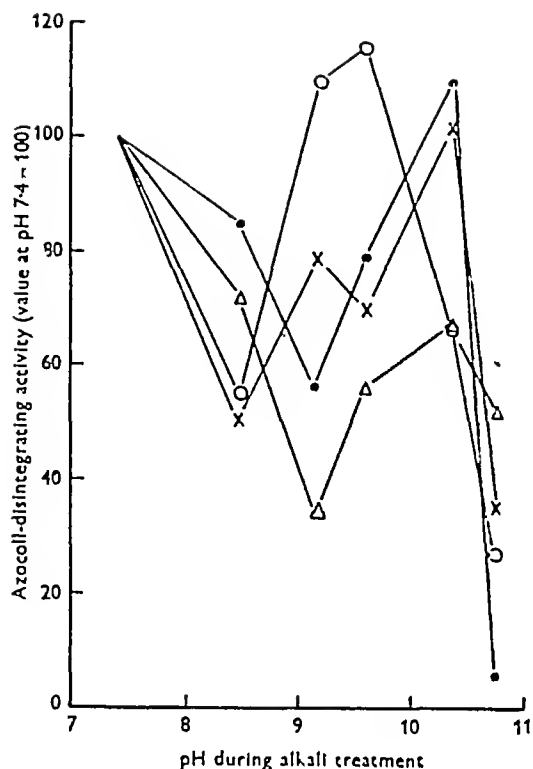


Fig 1 Azocoll-disintegrating activity after alkali treatment (a) pH changes effected by dialysis against borate buffers (Δ - Δ -) (b) pH changes effected by addition of 0.1N NaOH, (1) heated at 37° for 0.5 hr, \circ - \circ -, (2) left overnight at c 2°, -x-x-, (3) left 7 days at c 2°, \bullet - \bullet - pH readjusted to 7.4 with 0.1N-HCl before testing

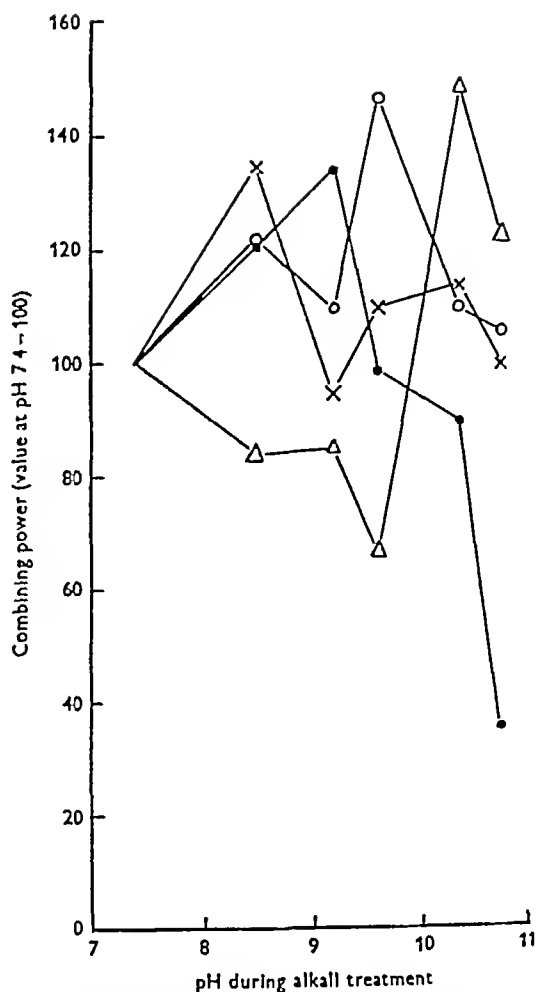


Fig 3 Apparent combining power using azocoll as indicator after alkali treatment Symbols as in Fig 1

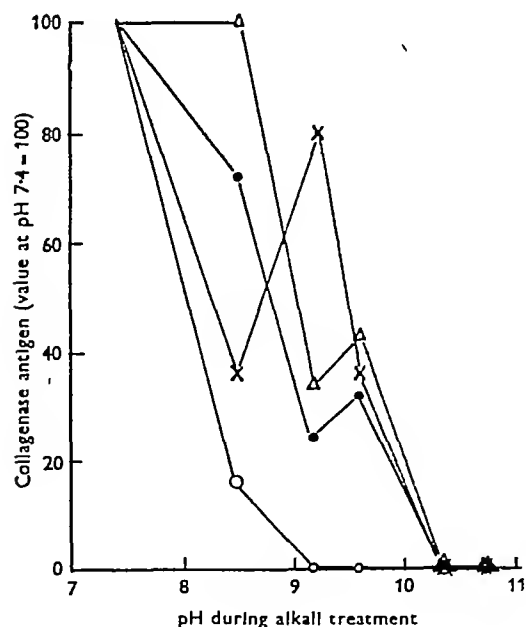


Fig 2 Collagenase antigen (combining power, muscle as indicator) after alkali treatment Symbols as in Fig 1

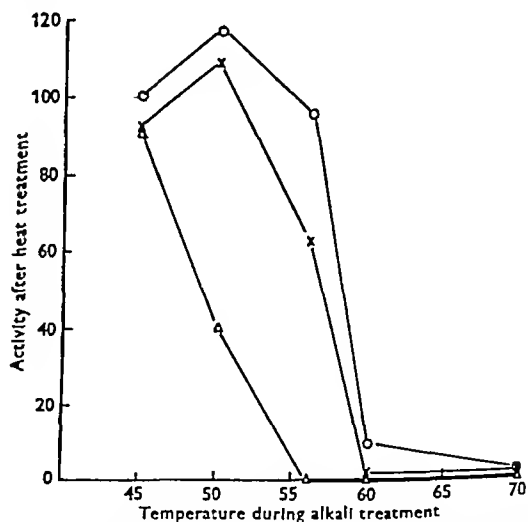


Fig 4 The effect on the activity of purified collagenase preparations of heating for 10 min at various temperatures Results are shown as a percentage of values obtained for a control solution kept at room temperature during the heating experiments Azocoll enzyme activity, -x-x-, antibody combining power (azocoll as indicator), \circ - \circ -, antibody combining power (muscle as indicator), Δ - Δ -

Solutions of purified collagenase preparations may undergo a similar change on long storage, since one such preparation after more than 2 years' storage at $c 2^\circ$ had lost most of its muscle-disintegrating activity while retaining its ability to disintegrate azocoll. Duthie & Lorenz (1948) had previously made a similar observation, old preparations of purified collagenase which had lost their true collagenase activity still possessed high gelatinase activity.

The effect of alkali treatment on the ability of enzyme preparations to disintegrate hide powder and to split gelatin The disintegration of commercial hide powder by an enzyme preparation which had been dialyzed at pH 10.2 was compared with that of the corresponding preparation before treatment, using the method described by Bidwell & van Heyningen (1948). The enzyme preparations were diluted to equivalent activities against azocoll. Over the range of pH tested (4.3–8.0) the amount of hide powder brought into solution by the two preparations was very nearly the same. The preparations were almost inactive at pH 4.3, but from pH 5.0 to 8.0 there was little variation in activity. Controls were run with heat-inactivated enzyme preparations as usual.

Experiments using enzyme preparations which had been dialyzed at pH 10.2 were made on gelatin, using the non-precipitable nitrogen technique (Bidwell & van Heyningen, 1948) and also the titration of liberated $-\text{NH}_2$ and $-\text{COOH}$ groups.

Five ml. enzyme preparation (a) or (b) (see below), or the corresponding heat-inactivated enzyme for controls, were pipetted into 40 ml. gelatin (0.5% (w/v) in borate buffer pH 7.2) at 37° , incubated 1 hr. at 37° , then 5 ml. phosphotungstic acid (5% (w/v) in $\text{N-H}_2\text{SO}_4$) added, shaken, filtered after 5 min. through Whatman no. 3 papers and total N determinations carried out on filtrates. Enzyme preparation (a) was a purified collagenase preparation which had not been treated with alkali. Enzyme preparation (b) was a similar preparation after dialysis against borate Na_2CO_3 buffer at pH 10.2.

The activity of the two preparations against azocoll was approximately the same (5 Q units/ml.), in the experiment with gelatin preparation (a) gave 5.69 mg. N/50 ml. filtrate, (b) gave 3.69 mg. N/50 ml. filtrate.

The results show that the alkali-treated preparation was still active against gelatin, but for a given activity against azocoll it appeared to be rather less active against gelatin than the untreated preparation. Both preparations liberated equivalent amounts of $-\text{NH}_2$ and $-\text{COOH}$ groups. The results on gelatin may be complicated by the possibility that part, at least, of the gelatin-splitting activity of purified enzyme preparations may be due to yet another enzyme, neither collagenase nor the enzyme formed on alkali treatment (Bidwell & van Heyningen, 1948, and unpublished results).

Some of the earlier work on substrate specificity was repeated, using enzyme preparations before and

after treatment at pH 10.2, which were highly active against azocoll. The action on casein and urea-denatured haemoglobin was tested by a method based on that of Anson (1938), but the incubation was carried out at 37° for 0.5 hr. The untreated preparations showed only a very slight trace of activity, the alkali-treated preparations were completely inactive.

DISCUSSION

It seems reasonable to reserve the name 'collagenase' for an enzyme capable of disintegrating 'native collagen', but the decision as to what is, or is not, 'native collagen' is a matter of some difficulty (see, for example, the discussion by Kanagy, 1947). Oakley, Warrack & Warren (1948) showed that filtrates of *Cl. welchii* types B and E (Bosworth, 1943) contain a substance capable of disintegrating azocoll but not muscle or collagen paper. The foregoing experiments support their view that ability to disintegrate hide powder (or azocoll) cannot be used as a specific test for true collagenase activity (see also Todd, 1947), but providing the latter is established by other means, azocoll is still useful as a substrate in quantitative estimations. At present, in the absence of evidence to the contrary, we regard as a true collagenase an enzyme capable of disintegrating muscle and collagen paper in the way described by Oakley *et al.* (1946), as well as bringing about solution of azocoll and splitting gelatin.

The experiments cited above showed that true collagenase (active against muscle and azocoll) began to be unstable in the cold at pH 8.5–9.0 and was completely destroyed at pH 10.3. On warming to 37° destruction was greatly accelerated, so that after 30 min. at 37° , even at pH 8.5 more than 80% was destroyed. It is suggested that a second enzyme (inactive against muscle, but active against hide powder and azocoll) appears at the higher pH values, the formation being slow in the cold at pH 9 and only considerable at pH 10. At still higher pH values (beyond 10.5) this enzyme also is destroyed. Heating at 37° speeds up both the formation and destruction of this postulated second enzyme, so that minimum and maximum azocoll-enzyme activities are observed at rather lower pH values than when the treatment is carried out in the cold. Heating to rather higher temperatures ($c 45$ – 50°) at pH 7.4 has an effect which appears to be similar to that of alkali treatment.

Since antisera, produced in response to the injection of culture filtrates of *Cl. welchii* type A, contain antibody to this second enzyme, an antigen capable of stimulating its production must be present in toxic filtrates which have not been alkali-treated. This does not necessarily imply that crude culture filtrates contain the second enzyme in an active form since the zymogen may itself be able to

stimulate production of antibody capable of neutralizing the action of the enzyme

Walburn & Reymann (1933, 1934) investigated the gelatinase of *Cl welchii* (presumably type A) and produced evidence to show that the enzyme had two pH optima—at c 6.0 and 8.0 respectively, with a minimum at c pH 7.5. The enzyme was incubated with the gelatin substrate at 37° for as long as 18 hr. They defined 1 enzyme unit as that quantity of enzyme which had digested the gelatin to such an extent that the mixture had just lost the capacity to form a solid gel on cooling under standard conditions. Walburn & Reymann tended to the view that culture filtrates of *Cl welchii* contain two gelatinases which have different pH optima. The results of the present work suggest that under the conditions they used, Walburn & Reymann may have been observing the resultant of several effects—the destruction at unfavourable pH of the 'primary' enzyme present in the original culture filtrate, the formation of the second enzyme and in addition the usual variation of enzyme activity with pH. The balance between the formation of the second enzyme at higher pH and the fact that its activity would appear from our work to be optimal at about neutral pH might very well explain Walburn & Reymann's observed second peak at pH 8.0. Their observation of a minimum at pH 6.7, i.e. lower than would be expected from the present work, may be connected with the fact that over this range they were using phosphate buffer which appears to be unfavourable to collagenase (Bidwell & van Heyningen, 1948). Jennison (1945, 1947) has shown that *Cl histolyticum* produces a true collagenase, so that the results of Walburn & Reymann (1934), showing two pH optima for the action of *Cl histolyticum* filtrates on gelatin, may also be due to the combination of several effects as discussed for *Cl welchii*.

Gonzalez (1946), in his investigation of the proteo-

lytic activity of toxic filtrates of gas gangrene bacteria, conducted his enzyme reactions at 50°, which appears from the present work to be in advisable, at least for *Cl welchii* filtrates, since this is the temperature at which destruction of the true collagenase and formation of the second enzyme was most marked.

The immunological relation between true collagenase and the enzyme present in alkali-treated preparations is being investigated by my colleagues Dr C. L. Oakley and Miss G. H. Warrack. The results of this work will be reported separately.

SUMMARY

1 The effect of mild alkali treatment and of heat on preparations of the collagenase of *Cl welchii* type A has been investigated. The activity of such preparations in disintegrating muscle and collagen 'paper' is readily destroyed at pH values higher than 8.5 even in the cold.

2 The effect of alkali treatment or of heating on the activity of these preparations in disintegrating hide powder and azocoll did not run parallel to the effect on the muscle-disintegrating activity.

3 It is suggested that, as a result of treatment at pH 9–10, or heating to c 50° for 10 min, a second enzyme is formed, derived either from collagenase or some other precursor present in culture filtrates.

4 This second enzyme disintegrates hide powder and azocoll, but is not a true collagenase, since it is without action on collagen paper and muscle. It is destroyed at pH values beyond 10.5 or by heating to 60° for 10 min.

I am grateful to Miss G. H. Warrack for many of the immunological tests, to Mr G. A. Spiby and Miss Beryl M. Sievwright for technical assistance, and to Dr E. S. Duthie, Dr C. L. Oakley and Miss G. H. Warrack for helpful discussions.

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The Sedimentation Constant, Diffusion Constant and Molecular Weight of Lactoglobulin

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In a previous paper (Cecil & Ogston, 1948) we have described experiments directed to improving and testing the accuracy of the ultracentrifuge, using lactoglobulin as a test substance. We satisfied ourselves that we could measure sedimentation constants correct to 0.5%. Lactoglobulin was used as a working substance in those experiments, we describe here some further experiments on its sedimentation and diffusion.

EXPERIMENTAL

Materials Three preparations of lactoglobulin were made from different samples of milk. Preparations 1 and 2 were made by the method of Palmer (1934). Preparation 3, made by a combination of the methods of Palmer and of Sørensen (1939), was found to be as homogeneous as preparations 1 and 2, but was obtained in greater yield and with less labour. The method was as follows.

Fresh milk, warmed to 30°, was cleared of cream by means of a de Laval centrifuge, using the 38 mm gravity ring. 39.2 g of solid $(\text{NH}_4)_2\text{SO}_4/100$ ml of milk were added. The precipitate, containing the casein and some whey globulins, was granular and filtered easily. A further 11.6 g $(\text{NH}_4)_2\text{SO}_4$ were added per 100 ml of filtrate, and the precipitate, after leaving to settle overnight, was then easily separated by filtration. It was dissolved in the minimal volume of water. Dialysis at pH 5.8 was found to give no precipitate, the pH was, therefore, adjusted directly to 5.2 by dialysis against acetate buffer. Dialysis against glass distilled water then led to crystallization.

Recrystallization was in all cases performed by dissolving the crystals in dilute NaCl solution and dialyzing against glass-distilled water. Insoluble material, when present, was removed by centrifuging and any adjustment of pH required was obtained by dialysis against buffer. The preparation of solutions and determination of protein concentration have been described in the previous paper (Cecil & Ogston, 1948).

Sedimentation measurements These were made over a range of concentrations using preparation 1, in 0.1 M NaCl, 0.1 M Na acetate and 0.04 M acetic acid, and measurements were made also in buffers containing 0.1 M NaCl, 0.1 M Na acetate and 0.01 M and 0.2 M acetic acid respectively. Assuming a pK of 4.74 for acetic acid, the pH values of these buffers were 5.14, 5.74 and 4.44. All measurements were made by the new standard procedure, and the results (Table 1) have been corrected for the error of the rotor thermocouple. Fig. 1 shows a plot of sedimentation constant against protein concentration, and includes values obtained by Johnston & Ogston (1946) on a different preparation of lactoglobulin.

Diffusion constant Diffusion runs were made in the pH 5.14 buffer with samples of three different preparations of lactoglobulin by the method of Coulson, Cox, Ogston & Philpot (1949). None of these proved to be quite homogeneous, by the criteria described by Ogston (1949) which were based on the relative positions of the interference bands arising from the diffusion boundary, in spite of exhaustive recrystallization and dialysis against buffer. However, Ogston (1949) has shown that it is possible to correct for the effects of heterogeneity on the apparent diffusion constant. Table 2 gives the apparent and corrected diffusion constants obtained in buffer at 20°, and the values of the diffusion constant corrected for the effect of buffer salts ($D_{20} \text{ corr}$).

RESULTS AND DISCUSSION

The sedimentation constant The mean value of $S_{20}(\text{corr})$ (Cecil & Ogston, 1948) for a protein concentration of 1 g/100 ml was 2.81×10^{-13} , extrapolation to zero concentration gives a value of 2.83×10^{-13} (Fig. 1). These values agree with previous measurements on another preparation of lactoglobulin made in this laboratory, but they disagree seriously with the results of Pedersen (1936).

Pedersen obtained mean values of 3.12×10^{-13} in the range 'pH 5.2-7' and 2.95×10^{-13} in the range 'pH 1-5' at concentrations of 1 g/100 ml and less. He does not give the exact compositions of his buffers, but in describing diffusion measurements, he quotes 5.0 as the pH of a buffer containing 0.2 M sodium chloride, 0.038 M sodium acetate and 0.012 M acetic acid. On this scale, our 'pH 5.14' buffer would have a pH of 4.9. This difference stresses the inconvenience of quoting pH values without giving the exact compositions of the solutions used (see Ogston, 1947). However, since we have found a fall of sedimentation constant on lowering the pH and no increase on raising it, it appears that our 'pH 5.14' falls within the range of conditions 'pH 5.2-7' of Pedersen, and that our value of the sedimentation constant is, therefore, to be compared with his value of 3.12×10^{-13} .

The very large difference between these results—nearly 10%—requires explanation. It might possibly arise from differences in the samples of lactoglobulin used by us and by Pedersen. Thus Bull (1946a) has found erroneous values for the surface pressure of lactoglobulin which was recrystallized after dissolving the crystals in dilute

Table 1 Variation of S_{20} with protein concentration and pH

Run	Conc of lactoglobulin (g/100 ml)	pH	Cell (mm)	$S_{20}(\text{corr}) \times 10^{13}$	Standard deviation of $S_{20}(\text{corr}) \times 10^{13}$
584	0.28	5.14	12	2.836	0.009
585	0.28	5.14	12	2.821	0.009
583	0.53	5.14	12	2.823	0.006
*	1.0	5.14	12	2.809	0.014
592	1.52	5.14	3	2.762	0.014
591	2.11	5.14	3	2.759	0.013
590	2.65	5.14	3	2.700	0.004
586	c 1.1	5.74	12	2.776	0.011
594	1.0	4.44	12	2.723	0.009

* From Cecil & Ogston (1948)

sodium hydroxide, the history, in this respect, of Pedersen's sample is not quoted, our samples were never exposed to a pH higher than that of native fresh milk

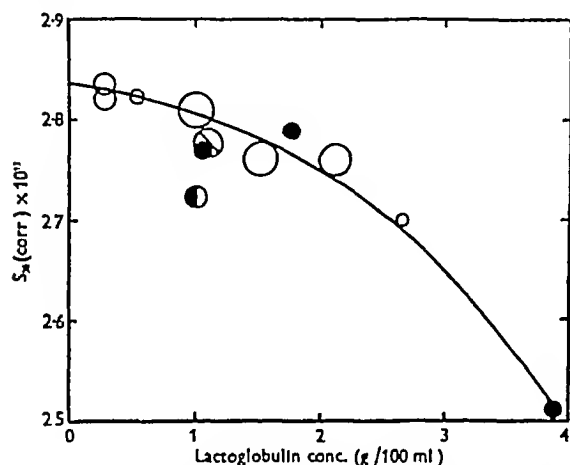


Fig 1 Fully corrected values of $S_{20}(\text{corr})$ against protein concentration. Filled circles: data of Johnston & Ogston (1946), corrected for effects of hydrogen pressure and thermocouple error. Open circles: data at pH 5.14, barred circle: value at pH 5.74, half-filled circle: value at pH 4.44. The radii of these circles are the estimated standard deviations.

On the other hand, the difference might arise from errors of one or both measurements. This explanation is supported by the result of a measurement kindly

made for us on one of our solutions (1 g/100 ml in the pH 5.14 buffer) by Mr C. J. Bradish, using the Svedberg ultracentrifuge at the Lister Institute, from which he obtained a value of $3.05 \pm (\text{s.d.}) 0.04 \times 10^{-13}$ for $S_{20}(\text{corr})$, in agreement with Pedersen's values. The discrepancy between this value and ours obtained on the same solution cannot be accounted for by errors in the measurement of speed. It therefore seems likely that the thermocouple readings in the Lister Institute ultracentrifuge are 2.5° lower than ours and, in our view, 3.5° below the actual cell temperature, and that a similar error has affected the Uppsala measurements. We have given reasons for believing (Cecil & Ogston, 1948) that we know the error of our rotor thermocouple (-1.0°) to within 0.1° and that no other systematic error affects our results.

The diffusion constant The values which we have obtained (Table 2) are not quite as consistent as might be desired, although some of the variation is due to variation of the degree of homogeneity in different samples, for which correction has been made, this correction in no case amounts to more than 2% and is believed to be a reliable one. The mean value of $D_{20}(\text{corr})$ at 1 g/100 ml is 7.70×10^{-7} and at infinite dilution 7.82×10^{-7} . These values differ considerably from those of Polson (1939) who obtained values of $D_{20}(\text{corr})$ between 7.10 and 7.27×10^{-7} at a concentration of 1 g/100 ml. Our method (Coulson *et al.* 1948) has given values in good

Table 2 Variation of D_{20} with concentration of lactoglobulin

Preparation	Conc of lactoglobulin (g/100 ml)	Apparent $D_{20} \times 10^7$	Corrected $D_{20} \times 10^7$	$D_{20}(\text{corr}) \times 10^7$
1	0.96	7.15	7.22	7.57
2	1.16	7.29	7.10	7.45
2	1.01	7.42	7.35	7.71
2	0.50	7.40	7.32	7.68
2	0.25	7.55	7.47	7.84
2	0.18	7.48	7.41	7.77
3	1.0	7.57	7.41	7.77
3	1.0	7.46	7.38	7.74

Mean value at 1 g/100 ml

 7.70 ± 0.09

Value extrapolated to zero concentration

7.82

agreement with those obtained by the method of Lamm (used by Polson), not only for glycine but for material of much lower diffusion constant. It seems likely, therefore, that the difference may depend on the sample of lactoglobulin and on the method of its preparation.

The molecular weight Estimates of the molecular weight of lactoglobulin by various methods range from 33,000, by X-ray diffraction on wet crystals of the orthorhombic form (McMeekin & Warner, 1942), to about 42,000, by sedimentation velocity and diffusion (Pedersen, 1936). The more reliable determinations by X-ray diffraction on dry crystals gave values about 36,000 (McMeekin & Warner, 1942). Pedersen's (1936) measurements on sedimentation equilibrium gave a mean value about 39,000, the variation of the value obtained with the method of computation suggests that the material used by him (and by Polson, 1939) was not homogeneous. Measurements of osmotic pressure have given values of 37,800 (Gutfreund, 1945), $37,300 \pm (s.d.) 300$ (Johnston & Ogston, 1946) and $35,020 \pm (s.d.) 140$ (Bull, 1946*a*). Bull (1946*b*) obtained a value of $2 \times 17,100$ from measurements of surface pressure.

The present values of the sedimentation and diffusion constants, using Pedersen's (1936) value of 0.751 for the partial specific volume, give values

of 35,600 at 1 g/100 ml and 35,400 at infinite dilution.

This wide variation of values is unsatisfactory. Errors may in some cases (as that of the sedimentation constant) have arisen from technical factors in the measurements, in others, variation may have been due to the treatment of the material during preparation, as is suggested by the data of Pedersen (1936) and the findings of Bull (1946*a*). It is difficult to decide which is the true value for the molecular weight, the most reliable determinations by X-ray diffraction favour a value near to 36,000, and our measurements on material which was very nearly homogeneous support this. The possibility exists, however, that lactoglobulin is not a material whose physical constants have unique values.

SUMMARY

1. Measurements of the sedimentation and diffusion constants of lactoglobulin are described. The values, extrapolated to infinite dilution, are $S_{20}(\text{corr}) = 2.83 \times 10^{-13}$ and $D_{20}(\text{corr}) = 7.82 \times 10^{-7}$, these differ considerably from other data but combine to give a molecular weight of 35,400. Other reliable values of the molecular weight agree with this.

2. The causes of differences between measured values of the constants of lactoglobulin are discussed.

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The Estimation of Peroxidase Activity

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The standard method for the estimation of the catalytic activity of peroxidase is that initially published by Willstätter & Stoll (1918). They allowed a measured quantity of the enzyme preparation dissolved in 2 l. distilled water to react with fixed amounts of hydrogen peroxide and pyrogallol for exactly 5 min. The reaction was then stopped by addition of sulphuric acid and the yellow purpurogallin transferred to ether in a separating funnel. The concentration of the ethereal solution was esti-

mated by comparison with standard purpurogallin solutions. The activity of the enzyme, Purpurogallin number (*P.N.*) or *Purpurogallinzahl* (*P.Z.*) of Willstätter, was defined as the weight of purpurogallin in mg. formed by 1 mg. of the enzyme preparation. Willstätter & Weber (1926) showed that the *P.Z.* values obtained varied over a wide range with changes in the quantities of the ingredients and in the volume of solution used for the test. A similar method for estimation of *P.N.* was

used by Elliott & Keilin (1934), Keilin & Mann (1937) and by Theorell (1942)

Recently, Sumner & Gjessing (1943), having mentioned that the work on peroxidase in their laboratory was 'somewhat hampered by lack of a satisfactory means of determining its activity', have described a new method in which the reaction is carried out in 20 ml phosphate buffer. They state that the resulting values for activity were somewhat higher than those obtained by Willstätter's method. In the present work the effect of dilution and of buffer concentrations on the activity of different enzyme preparations has been investigated, and the

of Elliott & Keilin (1934) and Keilin & Mann (1937), and a number of fractions of intermediate activity obtained during the fractionation of the crude juice by the same and by other methods. The reagents used were of A.R. standard, the pyrogallol being resublimed, the use of glass distilled water was found to be essential. All experiments were carried out at room temperature ($c. 20^\circ$). The King photoelectric colorimeter was calibrated for purpurogallin by means of standard solutions of the twice recrystallized pigment in peroxide free ether using an Ilford blue filter no. 302.

Willstätter's *PZ* estimation was modified slightly, being carried out in 500 ml water in presence of 12.5 mg H_2O_2 and 1.25 g pyrogallol (Keilin & Mann, 1937). The amount of enzyme, added at zero time, was adjusted in order that not more than 10 mg purpurogallin were formed in 5 min. The yellow ethereal extract was dried with Na_2SO_4 before being examined in the colorimeter.

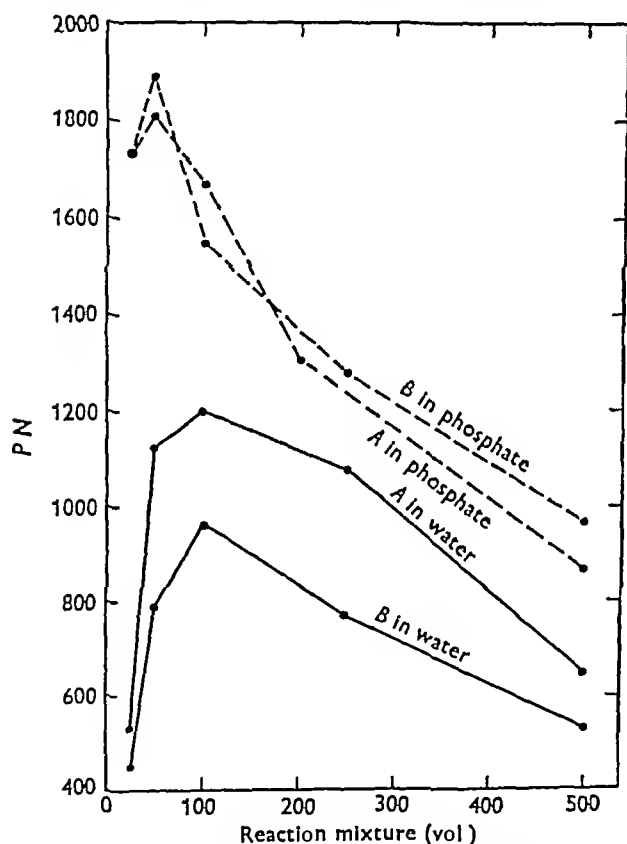


Fig 1 Effect of varying the volume of the reaction mixture on the activity of two highly purified peroxidase preparations, A and B, in water and in 0.07M-phosphate buffer pH 5.9. Reaction carried out in presence of 12.5 mg H_2O_2 and 1.25 g pyrogallol at 20°

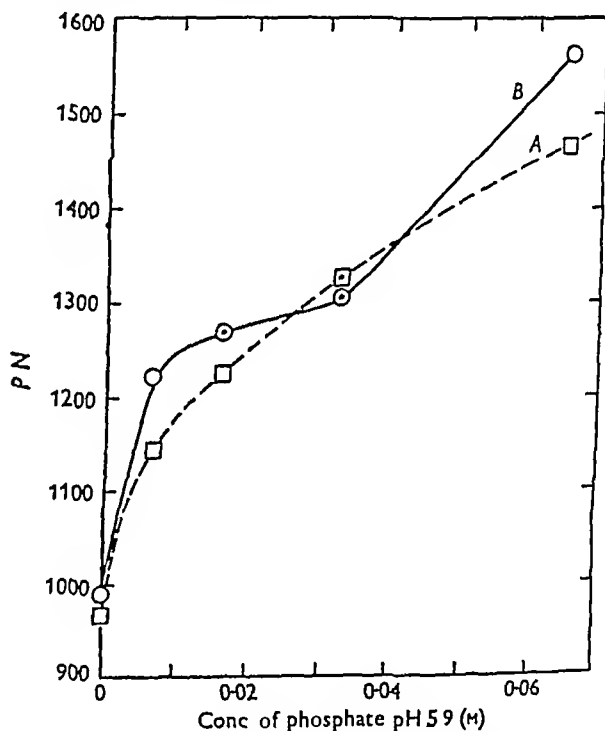


Fig 2 Effect of different concentrations of phosphate buffer on the activity of two highly purified peroxidase preparations, A and B. Reactions carried out in a volume of 100 ml in presence of 12.5 mg H_2O_2 and 1.25 g pyrogallol at 20° and pH 5.9

method devised by Willstätter and his co-workers compared with that of Sumner & Gjessing. The colorimetric methods have also been compared with a new manometric method based upon the formation of carbon dioxide during the catalyzed oxidation of pyrogallol.

EXPERIMENTAL

Methods

Enzyme preparations of widely differing purity were examined including the crude press juice of horse radish root, several highly purified preparations isolated by the methods

Influence of volume of reaction mixture and of phosphate buffer on the results obtained by Willstätter's method

Willstätter carried out his purpurogallin test at high dilutions in order to prevent destruction of enzyme by H_2O_2 , but, as Sumner & Gjessing (1943) have shown, high enzyme activity can be obtained in more concentrated solutions. Fig 1 shows the fluctuation in the *P N* values of two highly purified enzyme preparations observed when the total volume of the reaction mixture was varied between 25 and 500 ml. The conditions of the test were otherwise as described above. A maximum value for enzyme activity was

obtained in 100 ml water. When purified peroxidase preparations are used the concentration of protein in the test reaction is very low, and under these conditions denaturation may occur (Cohn & Edsall, 1943). In the presence of salts, which will increase the stability of the highly diluted protein and at the same time control the pH of the solution on which the reaction velocity is very dependent, higher and more reproducible values for P/N might be expected. Addition of phosphate buffer pH 5.9 to give a final concentration of 0.07M in the experiments just described yielded considerably higher figures for enzyme activity and the optimum volume was about 50 ml (Fig. 1). The effect of changing the concentration of phosphate, while maintaining the pH at 5.9 and the volume at 100 ml, is shown in Fig. 2 where the P/N is seen to increase with concentration of buffer. Thus, while Willstätter's conditions are far removed from the optimum, those of Sumner & Gjessing are very close. The risk of destruction of enzyme by high concentration of H_2O_2 has, therefore, been exaggerated by Willstätter: it is not until the volume of the reaction mixture falls below 100 ml that the destruction becomes considerable while, in presence of phosphate buffer, the harmful effect of H_2O_2 is greatly reduced.

A comparison of P/N determinations by the methods of Willstätter and of Sumner & Gjessing

The data on peroxidase activity in the literature are almost exclusively expressed in terms of Willstätter's P/Z . It is, therefore, important that the quantitative relationships between this and any new method should be determined in order that the values obtained by the new method may be translated to Willstätter's scale. With this object in view the comparative measurements summarized in Table 1

Table 1 *A comparison of the methods of Willstätter and of Sumner & Gjessing for the determination of enzyme activity of different peroxidase preparations*

Sample	Willstätter P/N	Sumner & Gjessing P/N	P/N ratio
No. 1 (purified)	735	1080	0.68
No. 2 (purified)	508	1460	0.35
Purpurogallin formed, mg/ml peroxidase preparation			
Horse radish press juice	68	100	0.68
Horse radish press juice, filtered	107	203	0.53
Two fractions isolated from press juice	280 83	810 228	0.35 0.36

were made. Sumner & Gjessing's method is as follows: 2 ml. 5% pyrogallol, 2 ml. 0.5M phosphate buffer pH 6.0, 15 ml. water and 1 ml. 1% H_2O_2 are mixed in a 125 ml. Erlenmeyer flask at 20°. Suitably diluted peroxidase (1 ml) is added, and after 5 min the reaction is stopped by addition of 1 ml. 2N- H_2SO_4 . The purpurogallin is extracted with ether and estimated in a colorimeter. All the determinations in Table 1 were made in duplicate, when agreement within experimental error was obtained. The recurrence of two values of the P/N ratio (last column) is striking but inexplicable, since each ratio (0.68, 0.35) was observed in the cases of both pure and crude preparations.

Manometric methods for estimation of peroxidase activity

The course of the oxidation of pyrogallol to purpurogallin was studied by Willstätter & Heiss (1923). According to them the overall reaction is



hence the formation of 1 mg purpurogallin should give rise to 102 μ l CO_2 . Experiments were carried out at 20° in Barcroft differential manometers with flasks which were fitted with side bulbs for the delivery of acid. The concentrations of the ingredients in the right hand flasks of the manometers were similar to those used in the method of Sumner & Gjessing for P/N determination, but the volume was reduced from 20 ml to 2.3 ml. Thus the right-hand flasks received 0.2 ml. 5% pyrogallol, 0.5 ml. 0.25M-phosphate buffer pH 5.9 and 1.5 ml. water. In a dangling tube, suspended from the central tube of the flask, was placed 0.1 ml. peroxidase preparation while the side bulb received 0.5 ml. 20% H_2SO_4 . The left-hand flasks received all the reagents except peroxidase.

In order to rule out the possibility of O_2 uptakes by crude enzyme preparations the flasks were evacuated and re-filled with N_2 containing 5% CO_2 . With purified preparations the same results were obtained in this gas mixture as in air. The flasks were equilibrated in the manometer bath for 10 min. and after closing the taps the peroxidase was

Table 2 *Comparison of manometric and colorimetric methods for estimation of peroxidase*

(Preparation no. 1 (0.0016 mg) and press juice (1.88 mg dry wt) were used in these estimations.)

Enzyme	A. Estimations of purpurogallin formed from pyrogallol by peroxidase	
	Manometrically by CO_2 production (mg)	Colorimetrically (mg)
Preparation no. 1	1.97	2.09
Filtered press juice of horse radish root	2.15	2.25
	B. Estimations of peroxidase activity by two methods	
	Manometrically by CO_2 production (P/N)	Colorimetrically (P/N)
Preparation no. 1	1250	1370
Filtered press juice of horse-radish root	1.34	1.28

added. At 5 min the CO_2 output was recorded and the acid immediately added from the side bulbs. The purpurogallin formation was calculated from the CO_2 evolved, and was checked by colorimetric examination of the ethereal extracts of the solutions in the flasks. As is seen in Table 2A there is good agreement between the results. In further experiments the P/N of a preparation was estimated in two samples by the manometric method and also by the method of Sumner & Gjessing. Both crude and pure peroxidase fractions yielded results in good agreement (Table 2B).

Further experiments on CO_2 production during the formation of pyrogallol were carried out in a Brinkman apparatus as used by Meldrum & Roughton (1934) for the estimation of carbonic anhydrase. In this method the reaction mixture is shaken very rapidly in order to maintain equilibrium between the gas phase and the dissolved gases during rapid reactions. A modified apparatus was used for this work in which the manometer was replaced by a manometric capsule incorporating a very thin diaphragm. Movements of the latter were recorded by means of an optical lever and mirror. With this arrangement there is virtually no time lag between the formation of a gas and its recording by the optical lever. Reaction mixtures of the type used in the Barcroft manometers were tested in this apparatus, and, with rapid shaking, a close proportionality between quantity of enzyme taken and rate of CO_2 evolution was observed over a period of 40 sec. After this time denaturation of the enzyme is likely to occur.

The experiments just described show that manometric determinations of peroxidase activity are possible. * The close agreement between this method and the colorimetric method as modified by Sumner & Gjessing enables peroxidase activity to be expressed in terms of Q_{CO_2} and hence allows $P.N$ values to be converted to the commonly used Q notation. Thus $P.N = 1$ corresponds to $Q_{\text{CO}_2} = 102 \times 12 = 1224$.

Observations on the stability of peroxidase preparations

Experience gained during the present investigation confirms the observations of Willstätter and of other workers on the large and unaccountable variations of about 30 % which are obtained when the $P.N$ of a peroxidase preparation is tested periodically. A possible explanation of these variations was the lack of buffering capacity in the Willstätter

* The manometric method described above is fundamentally different from that devised by Altschul & Karon (1947) which consists of the estimation of unused H_2O_2 in terms of O_2 liberated by catalase.

method, but an investigation, in which the $P.N$ of a very active peroxidase was tested at intervals during 7 weeks in presence of phosphate by the method of Sumner & Gjessing, did not confirm this view. In spite of the control of pH the $P.N$ varied haphazardly between 1160 and 1780 although duplicate simultaneous determinations were in good agreement. These experiments confirm the view that such variations are due not to any changes in the peroxidase preparation, which would give rise to a progressive change in $P.N$, but rather to minute traces of impurities, possibly heavy metals, in the reagents.

SUMMARY

1 The methods for the determination of peroxidase activity based upon the catalyzed oxidation of pyrogallol to purpurogallin by hydrogen peroxide have been re-examined.

2 The effects of varying concentrations of reactants and of phosphate buffer have been investigated with a view to the development of a method giving more reproducible results.

3 A manometric method, based upon the evolution of carbon dioxide during the oxidation, has been devised which gives results in good agreement with more recent methods involving colorimetric determinations of purpurogallin. It thus becomes possible to express the activity of peroxidase preparations in terms of Q_{CO_2} .

4 The periodic fluctuations in apparent peroxidase activity of enzyme preparations, the origin of which still remains obscure, have been confirmed.

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The Fate of Certain Organic Acids and Amides in the Rabbit

6 NITROBENZOIC ACIDS AND AMIDES

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Studies *in vitro* (Bray, Jamos, Ryman & Thorpe, 1948a) have shown that rabbit-liver extracts are able to hydrolyze all three nitrobenzamides. The para isomer is most readily attacked, the average hydrolysis being 82 %, under the same conditions the meta and ortho isomers are hydrolyzed to the extent of 31 and 23 %, respectively. It was, therefore, of interest to study the metabolism of those compounds *in vivo*, comparing them with the corresponding nitrobenzoic acids. The literature contains little information concerning the metabolism of these acids in the rabbit, though there are some accounts of investigations upon other animals. As early as 1851 Bertagnini found that *m*-nitrobenzoic acid was excreted as the corresponding hippuric acid by the dog. Quick (1932) included the three nitrobenzoic acids in an extensive study of the glycine and glucuronic acid conjugation of nuclear substituted benzoic acids in the dog and found that the meta and para isomers, but not the ortho, were conjugated with glycine to a considerable extent and that all three were excreted to some extent as ester glucuronides.

Nitrobenzaldehydes and nitrotoluenes are oxidized to the corresponding nitrobenzoic acids *in vivo*. *o*-Nitrobenzaldehyde is excreted as *o*-nitrobenzoic acid in man (Sherwin & Hynes, 1921), rabbit (Cohn, 1893) and dog (Sieber & Smirnow, 1887). *m* and *p*-Nitrobenzaldehydes give rise to the corresponding nitrobenzoic and hippuric acids in man (Sherwin & Hynes, 1921) and dog (Sieber & Smirnow, 1887). Cohn (1893) also found that *m*-nitrobenzaldehyde was converted to some extent to *m*-nitrohippuric acid in the rabbit, though conjugation with glycine did not appear to take place with the para isomer in this animal. Jaffe (1878-9) found that *o*-nitrotoluene was converted in the dog to the corresponding benzyl alcohol and benzoic acid, the latter being excreted unconjugated, *p*-nitrotoluene (Jaffe, 1874) was oxidized to *p*-nitrobenzoic acid which was excreted as its glycine conjugate.

A further change which aromatic nitro acids may undergo *in vivo* is reduction of the nitro group. Cohn (1893, 1894) was the first to observe this, isolating *m*-acetamidobenzoic acid as a metabolite of *m*-nitrobenzaldehyde and *m*-nitrobenzoic acid in the rabbit, he also isolated *p*-acetamidobenzoic acid as a metabolite of *p*-nitrobenzaldehyde. The extent of

reduction was greater with the meta aldehyde than with the corresponding acid. Sherwin & Hynes (1921) did not observe any reduction of nitrobenzaldehydes in man. More recently some results of a study of the reduction of nitrobenzoic acids in the rat, and *in vitro* by rat and mouse liver and kidney have been reported (Kohl & Flynn, 1941). These will be referred to later.

In the present study we have investigated the excretion of ether-soluble acid and diazotizable material by rabbits, after the administration of the nitrobenzoic acids and amides, and have also identified their principal metabolites.

METHODS

Diet and dosing. The rabbits used were does of approx. 3 kg wt. The diet of rabbit pellets has already been described (Bray, Ryman & Thorpe, 1947). The compounds were administered by stomach tube, the acids as solutions in NaHCO₃ and the amides, which were prepared by the action of NH₃ on the corresponding acid chlorides, as suspensions in water. All the acids and amides could be safely administered at a dose level of 0.1 g/kg, and in most cases doses of 0.2 g/kg caused no ill effects. Repeated administration of any of the compounds, however, resulted in death. *o*-Nitrobenzamide was the most toxic compound of the series and in some cases the administration of a single dose, even at the lower level, was fatal. Consequently, the number of experiments we were able to make with this compound was limited.

Estimation of ether soluble acid. The method was that used previously (Bray *et al.* 1947). The ether soluble material was titrated directly against 0.05N NaOH with thymol blue as indicator.

Estimation of diazotizable material. The method used was that of Bratton & Marshall (1939). The standard used for comparison was a solution of the corresponding aminobenzoic acid. The estimation was performed on the urine (a) as collected, (b) after acetylation, (c) after hydrolysis and (d) after reduction. Acetylation was effected by shaking together urine (10 ml) and acetic anhydride (1 ml) for 1 min, and allowing the mixture to stand for 30 min before estimating in the usual way. Control experiments showed that solutions of aminobenzoic acids of equivalent concentration were completely acetylated by this treatment, and it was therefore assumed that any colour produced by the diazotization and coupling of acetylated urine was due to hydroxylamine derivatives (cf. Rosenthal & Bauer, 1939). Hydrolysis was carried out as in the Bratton

& Marshall procedure Since the amounts of hydroxylamine derivatives detected were very small (see p 41), any changes they undergo on hydrolysis may be neglected, and the result obtained by estimation of hydrolyzed urine taken as indicating the total extent of reduction *in vivo* and, in conjunction with the result obtained from hydrolyzed urine, of the degree of acetylation Reduction was effected by treating the urine (10 ml) with HCl (4 ml, 2N) and granulated Zn (200 mg) in a boiling water bath for 1 hr Estimation on this solution gave the total excretion of nitro compound, whether reduced, acetylated or unchanged Reduction by TiCl_3 (Eckert, 1943) was found to give less satisfactory results Control experiments showed that *p* nitrobenzoic acid and amide were both reduced to the extent of 92% (average of 5 estimations, range 89–93%) This value could not be increased by, e.g. recrystallization of acid, increased reduction time or use of more Zn, and so the necessary correction factor was applied to the calculation of results The meta isomers were both quantitatively reduced *o* Nitrobenzoic acid was also quantitatively reduced, but it was found that *o* nitrobenzamide urines on diazotization and coupling gave a bluish red colour which could not be matched against any standard used, so that no quantitative diazo estimations were carried out

Estimation of reducing material This was carried out with unhydrolyzed urine by the method already described (Bray, Neale & Thorpe, 1946)

Estimation of ethereal sulphate The method of Folin (1905–6) was again used

RESULTS

Quantitative studies

Excretion of ether soluble acid The average normal values for individual rabbits lay between 692 and 807 mg/day (calculated as hippuric acid) The day to day variation in output for each rabbit was usually within $\pm 7\%$ of the mean Table 1 shows the percentages of doses excreted as ether soluble acid, calculated in all cases as nitrobenzoic acid A possible source of error is the fact that the 'extra' ether soluble acid does not consist solely of nitrobenzoic acid, though this undoubtedly constitutes the major part of it It can be seen from Table 2 that a portion of the dose of all six compounds studied is excreted as the corresponding aminobenzoic acid, partly free and partly acetylated. Acetamidobenzoic acids are extracted quantitatively under the conditions used here (cf Bray & Thorpe, 1948) The

Table 1 *Excretion of ether-soluble acid (calculated as nitrobenzoic acid) by rabbits after the administration of nitrobenzoic acids and amides*

<i>o</i> Compound			<i>m</i> Compound			<i>p</i> Compound		
Rabbit no	Dose (g/kg)	Dose excreted as ether soluble acid (%)	Rabbit no	Dose (g/kg)	Dose excreted as ether soluble acid (%)	Rabbit no	Dose (g/kg)	Dose excreted as ether soluble acid (%)
<i>(a) Acids</i>								
126	0.1	83	84	0.1	128	84	0.1	97
131	0.1	103		0.1	99		0.1	96
			109	0.1	109	109	0.1	125
				0.1	87		0.1	99
			126	0.1	110			
Averages		93			105			104
126	0.2	85	84	0.2	104	84	0.2	99
	0.2	96	109	0.2	79		0.2	107
131	0.2	83	126	0.2	109		0.2	108
				0.2	97	109	0.2	109
			131	0.2	110		0.2	88
Averages		88			100			102
<i>(b) Amides</i>								
126	0.1	29	84	0.1	48	84	0.1	120
131	0.1	23		0.1	60		0.1	89
			109	0.1	75	109	0.1	132
				0.1	73		0.1	90
			131	0.1	55			
Averages		26			62			108
126	0.2	19	101	0.2	55	84	0.2	96
131	0.2	10	109	0.2	43		0.2	104
				0.2	52		0.2	95
			126	0.2	71	109	0.2	91
			131	0.2	48		0.2	84
							0.2	85
Averages		15			54			93

Table 2 *Diazotizable material in rabbit urine after administration of nitrobenzoic acids and amides*

Rabbit no	Dose (g /kg)	Dose excreted as diazotizable material in urine (%)			
		As collected	After acetylation	After hydrolysis	After reduction
o Compound					
(a) Acid					
109	0.1	21	1.2	20	75
126	0.1	18	2.6	17	74
131	0.1	27	1.9	24	73
Averages		22	1.9	20	74
126	0.2	26	3.7	26	81
	0.2	19	4.3	16	79
131	0.2	24	1.5	23	76
Averages		23	3.2	22	79
m Compound					
(a) Acid					
84	0.1	8.5	1.9	14	85
	0.1	4.7	1.6	9	85
109	0.1	5.1	0.7	13	78
	0.1	4.1	0.4	11	81
126	0.1	4.7	1.1	9	90
Averages		5.6	1.1	11	84
84	0.2	3.9	0.9	8	74
109	0.2	5.1	0.6	11	78
131	0.2	3.9	0.6	12	90
Averages		4.3	0.7	10	81
(b) Amide					
84	0.1	3.4	1.7	26	73
	0.1	2.5	1.7	26	77
109	0.1	2.5	0.9	27	72
	0.1	2.2	1.1	29	81
131	0.1	1.8	1.5	24	73
Averages		2.5	1.4	26	75
101	0.2	2.4	0.7	27	75
109	0.2	2.4	0.6	24	80
	0.2	1.9	0.6	21	79
Averages		2.2	0.6	24	78
p Compound					
(a) Acid					
84	0.1	2.2	1.1	15	90
	0.1	1.6	0.9	13	96
109	0.1	1.5	0.9	9	86
	0.1	1.1	0.7	9	86
Averages		1.6	0.9	12	90
84	0.2	1.4	0.5	13	86
	0.2	1.0	0.5	11	100
109	0.2	0.9	0.4	9	86
	0.2	1.7	0.5	12	90
Averages		1.2	0.5	11	91
(b) Amide					
84	0.1	2.5	1.2	16	91
	0.1	1.4	0.8	15	97
109	0.1	2.2	1.1	13	88
	0.1	2.9	1.7	16	99
Averages		2.3	1.2	15	94
84	0.2	1.2	0.5	14	101
	0.2	3.0	1.0	14	97
109	0.2	1.5	1.1	15	102
	0.2	1.7	0.6	15	99
Averages		1.9	0.8	15	100

largest acetyl excretion is from *m* nitrobenzamide, and even here the error due to calculation of *m* acetamidobenzoic acid as nitrobenzoic acid would be less than 2% (low). Only about 90% of free aminobenzoic acids are extracted under the conditions used (pH 1.5–2.0, pH 4.0 is necessary for complete extraction (Bray, Lake, Neale, Thorpe & Wood, 1948b)). The greatest conversion to free aminobenzoic acid occurs in the ortho isomer (22%) thus the error due to unextracted aminobenzoic acid would be about 2.2% (low). Calculation of the extracted aminobenzoic acid (19.8%) as nitrobenzoic acid would give a value 4.3% high, so that the net error due to aminobenzoic acid would only be of the order of 2% (high), which is hardly significant, even in this extreme case. There is also the possibility in the meta and para isomers of conjugation with glycine, but qualitative studies, described below, led us to conclude that the amounts of nitrohippuric acid excreted are very small, if indeed such compounds are formed at all. It is probable, therefore, that the results given in Table 1 give a reliable indication of the relative amounts of the amides hydrolyzed, since the conditions used were identical for both acid and amide urines. This is least certain in the case of *o* nitrobenzamide in view of the more complex nature of its excretion products. As reported later, however, the only material isolated from acidified *o* nitrobenzamide urine under the conditions used for the estimation of ether-soluble acid appeared to consist of small amounts of unchanged amide and *o* nitrobenzoic acid. The hydroxylated metabolites appear to be largely conjugated with sulphuric or glucuronic acids, the resulting conjugates being not appreciably soluble in ether.

Excretion of diazotizable material. The results obtained are summarized in Table 2. As already stated, the standards used in the diazo estimations were the corresponding amino benzoic acids. The interpretation of the results for the meta and para isomers is straightforward since there is no significant difference between the degrees of reduction of the acids and their amides by Zn and HCl or between the intensities of the colours developed from them on diazotization and coupling (see Bray *et al.* 1948b). The results obtained with *o* nitrobenzoic acid similarly may be interpreted directly, but no values can be given for its amide, since most of the colours given could not be matched with either an anthranilic acid or an amide standard. This is due to the fact that *o* nitrobenzamide is hydroxylated to a considerable extent in the rabbit. The only colour which could be matched was that given by acetylated urine. The results corresponded to 1–2% of the dose.

Our results in general are similar to those obtained by Kohl & Flynn (1941) using rats. These investigators found that 21% of *o*, 8% of *m*- and 2.5–4.0% of *p* nitrobenzoic

acid was excreted as unconjugated amino compounds, the percentages of the doses of *m* and *p* nitrobenzoic acids excreted as acetamido compounds were 22 and 11–20 respectively. By reduction of the urines 72, 63 and 89–94% of the doses of the *o*, *m* and *p* acids were accounted for.

Excretion of ethereal sulphate. The only one of the compounds under investigation to cause an increase in the excretion of ethereal sulphate was *o* nitrobenzamide. In 3 experiments at dose levels of 0.1 and 0.2 g/kg 48, 49 and 46% of the doses were excreted conjugated with H₂SO₄. The average normal daily excretion of ethereal sulphate by individual rabbits ranged from 24–52 mg SO₃. The day-to-day variation in output for each rabbit was usually within $\pm 7\%$ (i.e. approx. 3 mg) of the mean.

Excretion of reducing material. The values obtained cannot be regarded as reliable since (a) the actual increases were very small compared with the variations which occur in the amounts of reducing material normally excreted, and (b) diazo estimations (Table 2) suggest the presence of small amounts of hydroxylamino compounds which would be reducing. Furthermore, there was considerable variation in the values obtained for each compound. The average values for the percentage of the dose excreted as apparent ester glucuronide were ortho acid 16% (range 6–25), amide 12% (range 11–13), meta acid 8% (range 0–17), amide 4% (range 2–5), para acid 3% (range 0–10), amide 8% (range 0–21). The amounts were too small for isolation. The average normal daily excretion of reducing material ranged from 211 to 270 mg (calculated as glucuronic acid).

Reduction of nitrobenzoic acids and amides by rabbit liver extracts in vitro. Solutions of the acids and amides (100 mg/100 ml) in phosphate buffer pH 7.4 were incubated at 37° with extracts of rabbit liver (4 ml extract/20 ml solution) prepared as described previously (Bray *et al.* 1948a). After 24 hr, samples of the digest (2 ml) were withdrawn, deproteinized with 10% trichloroacetic acid (8 ml) and the azotizable material estimated in the filtrate by the method of Bratton & Marshall (1939), using aminobenzoic acid standards. Table 3 shows the percentages of the compounds converted into diazotizable material under these conditions in 24 hr. The results obtained by Kohl & Flynn (1941) for the reduction of the acids by rat liver *in vitro* are also shown. The results obtained with *o* nitrobenzamide cannot be interpreted fully since it is not known whether the reduction product is *o* aminobenzoic acid or its amide, the extracts used being capable of hydrolyzing the amide (e.g. to an extent of 25% in the second experiment; see also Bray *et al.* 1948a). If the amino amide is formed the results are low, since that compound on diazotization and coupling gives only 68% of the colour given by a solution of anthranilic acid of equivalent strength (Bray *et al.* 1948b).

Table 3 Reduction of nitrobenzoic acids and amides by rabbit liver extracts in vitro at pH 7.4

Compound	Percentage reduction in 24 hr				Reduction by rat liver in 7 hr *
	Exp 1		Exp 2		
	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed	
<i>o</i> -Nitrobenzoic acid	2.9	2.2	2.8	2.8	1.43
<i>o</i> -Nitrobenzamide	5.2	5.2	4.7	5.1	
<i>m</i> -Nitrobenzoic acid	5.6	6.2	5.3	5.0	30.3
<i>m</i> -Nitrobenzamide	4.7	4.8	5.7	5.9	
<i>p</i> -Nitrobenzoic acid	3.4	4.2	3.2	3.8	28.2
<i>p</i> -Nitrobenzamide	4.1	4.1	5.8	4.2	

* Kohl & Flynn (1941)

Hydrolysis of the digests in most cases caused a slight increase (up to 0.8%) in the amount of diazotizable material present. We were not able to determine whether this was due to acetylation as the change is within the limits of experimental error. Klein & Harris (1938) found that acetylation of sulphanilamide occurred only with liver slices and not in brew. We ourselves (unpublished results) have found that the liver extracts used brought about the deacetylation of acetanilide, *o*, *m* and *p* acetotoluidides, *m* acetamidobenzoic acid and *p* hydroxyacetanilide (cf. Michiel, Bernheim & Bernheim, 1937).

Qualitative experiments

These were carried out in order to determine the nature of the main excretion products of the 6 compounds being studied. Continuous ether extraction of the acidified nitrobenzoic acid urines resulted in the isolation in all 3 cases of the unchanged acid in considerable amounts. They were recrystallized from water and their identity confirmed by comparison with authentic specimens. The mother liquors in every case gave a positive diazo reaction, but the compounds responsible were not isolated.

From *p* nitrobenzamide urines, *p* nitrobenzoic acid was isolated in yields similar to those obtained from the acid, which is in accordance with the quantitative finding that the carbamyl group in *p* nitrobenzamide is virtually completely hydrolyzed in the rabbit.

Ether extraction of acidified *m* nitrobenzamide urine led to the isolation and characterization of *m* nitrobenzoic acid. Extraction of the urine as collected (pH 8) gave *m* acetamidobenzoamide, m.p. 219°, mixed m.p. with an authentic sample (m.p. 220°), 219°. Yield, 100 mg from 2.5 g amide administered. This provides direct evidence that the carbamyl group in *m* nitrobenzamide is not completely hydrolyzed in the rabbit, and that the reduction product is the corresponding amino compound.

All attempts to isolate glycine conjugates of *m*- and *p*-nitrobenzoic acids were unsuccessful; similar results were obtained from experiments in which glycine (0.2 g) was administered along with the acids (0.4 g). It thus appears probable that *m*- and *p* nitrohippuric acids are not formed as metabolites of the nitrobenzoic acids in the rabbit. No evidence for the formation of azoxy, azo or hydrazo compounds was obtained.

As already mentioned, ether extraction of *o* nitrobenzamide urine as collected gave small amounts of the amide itself, and extraction of acidified urine gave very small amounts of *o* nitrobenzoic acid. The isolation of metabolites from hydrolyzed *o* nitrobenzamide urine was difficult on account of the low dose level which had to be used. A typical experiment was as follows: the 24 hr urine (4 l) of 12 rabbits which had received *o* nitrobenzamide (0.4 g each) was hydrolyzed by boiling for 30 min with conc. HCl (0.2 vol.), adjusted to pH 5 and continuously extracted with ether for 96 hr. The ether layer showed an intense blue fluorescence which persisted throughout this period. The ether soluble material consisted of a dark brown syrup containing some solid matter (extract A). The urine was reacidified as before, boiled for 1 hr and continuously extracted with ether for 48 hr to yield extract B, a brown syrup. The pH of the urine was then adjusted to 5 and extraction with ether continued for a further 96 hr; the ether layer again showed a blue fluorescence. In this way extract C was obtained.

Extract A. On the addition of a little ethanol the syrup dissolved and crystalline material (50 mg) remained which did not melt below 300° and gave no coloration with FeCl₃ and a negative diazo reaction. Boiling with conc. HCl did not liberate a diazotizable amino group, but gave a crystalline compound which appeared to be a hydrochloride (D). A solution of the crystals in water slowly deposited the free base as needles which had no m.p. but decomposed with volatilization at 360°. Analysis of the hydrochloride gave C, 46.3, H, 4.9, N, 11.7, Cl, 15.1%. The hydrochloride did not give a diazo reaction after reduction with Zn and HCl. Its properties were not compatible with those which would be expected from an azoxy, azo or hydrazo compound.

Addition of more ethanol to the syrup gave a further amount of solid material (50 mg) which decomposed at 220–240° and gave an intense purple brown coloration and precipitate with FeCl₃, a positive diazo reaction (a blue-violet colour developing slowly), a positive indophenol reaction and an intense blue fluorescence in ethereal solution. On acetylation it gave a crystalline compound, m.p. 235–236°. By comparison with authentic specimens it was shown that the compound isolated was 5 hydroxyanthranilic acid (cf. Bray *et al.* 1948b). The syrup which remained after the removal of the solid material gave a positive diazo reaction (reddish purple) and a purple brown coloration with FeCl₃. It was extracted with boiling water and from the aqueous extract needle-like crystals, m.p. 174°, were obtained (100 mg). These were shown to be *o* nitrobenzamide, since they did not depress the m.p. (174°) of an authentic sample. The colour reactions of the syrup remaining were unchanged. Unsuccessful attempts were made to isolate the metabolites present (probably, judging from the diazo colours, anthranilic and 5 hydroxyanthranilic acids or their amides) by treatment with water or organic solvents and by acetylation.

Extract B. The only compounds identified in this fraction were *o* nitrobenzoic acid (150 mg) and benzoic acid (200 mg), the latter presumably derived from hippuric acid.

Extract C. This contained some solid material which could be separated from the syrup present by treatment with ethanol; it was shown to be 5 hydroxyanthranilic acid. The ethanolic solution gave a brownish colour with FeCl₃ and a yellow coloration with HNO₂ which might have been due to the presence of an *o* aminophenol derivative such as 3 hydroxyanthranilic acid (cf. Bray *et al.* 1948b), but the amount was too small to permit of its identification.

In another experiment a further product was obtained in addition to those already described. This was an amorphous powder which did not melt below 300° and gave no coloration with FeCl₃ and a negative diazo reaction. On boiling with conc. HCl for 3 hr and evaporation of the resultant solution a crystalline compound was obtained which appeared to be identical with the hydrochloride D from extract A. Further evaporation of the mother liquor gave prisms giving positive diazo, FeCl₃ and indophenol reactions. These were identified as 5 hydroxyanthranilic acid hydrochloride. Found N, 7.6. Calc. for C₇H₈NO₃Cl N, 7.4%.

DISCUSSION

Table 4 summarizes the analytical results obtained. In view of the unreliability of the values for ester glucuronide (*q.v.*) ether-soluble acid excretion only has been given for the degree of hydrolysis of the

Table 4 Metabolites of nitrobenzoic acids and amides in the rabbit

Percentage of dose excreted as

Compound	Ether soluble acid	Amino compound (free)	Amino compound (acetylated)	Amino compound (total)	Hydroxylamine derivative	Ethereal sulphate	Total percentage excreted (by diazo)	Estimated percentage hydrolysis of amides*	Percentage hydrolysis by liver extracts†
<i>o</i> Nitrobenzoic acid	90	20	0	20	2.6	0	77	—	—
<i>o</i> Nitrobenzamide	21	—	—	—	—	48	—	21 (33)	23
<i>m</i> -Nitrobenzoic acid	103	4	7	11	0.9	0	83	—	—
<i>m</i> -Nitrobenzamide	58	2	23	25	1.0	0	77	58 (62)	31
<i>p</i> Nitrobenzoic acid	103	1	10	11	0.7	0	91	—	—
<i>p</i> Nitrobenzamide	99	2	13	15	1.0	0	97	99 (107)	82

* These estimated percentages do not include ester glucuronide values (*q v*) These are included in the values in parentheses
 † Bray *et al* (1948a)

amides in the rabbit, so that these estimates may be low. The figures in parentheses give the degree assuming all the reducing value to be due to ester glucuronide and are almost certainly too high. The results are of the same order as those obtained using rabbit-liver extracts (Bray *et al* 1948a). This agreement has been found in all the aromatic amides so far studied, as shown in Table 5, if allowance is

Table 5 Hydrolysis of nuclear substituted benzamides in the rabbit and by extracts of rabbit liver *in vitro*

Compound	Percentage hydrolysis	
	In intact animal	By liver extract (6) (at equilibrium)
Benzamide	100 (1)	45
<i>p</i> Nitrobenzamide	99 (2)	82
<i>m</i> Nitrobenzamide	58 (2)	31
<i>o</i> Nitrobenzamide	21 (2)	23
<i>p</i> Aminobenzamide	19 (3)	13
<i>m</i> Aminobenzamide	10 (3)	7
<i>o</i> Aminobenzamide	33-12 (3*)	9
<i>p</i> Hydroxybenzamide	4 (4)	6
<i>m</i> -Hydroxybenzamide	11 (5)	1
<i>o</i> Hydroxybenzamide	6 (5)	0

* An average is not given here owing to the wide difference in response to dose level

(1) Bray *et al* (1946)

(2) Present paper

(3) Bray *et al* (1948b)

(4) Bray *et al* (1947)

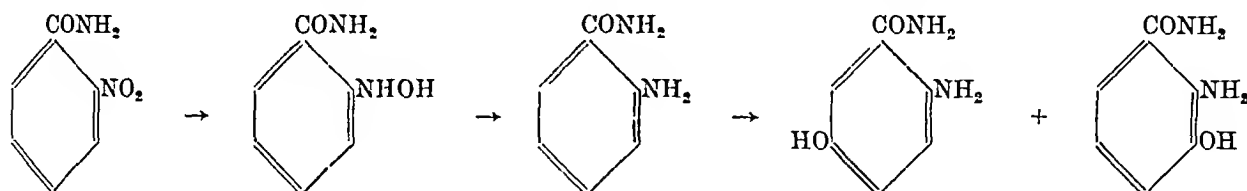
(5) Bray *et al* (1948c)

(6) Bray *et al* (1948a)

made for the essentially dynamic conditions which prevail in the living cell and which, in general, enable reactions to proceed further towards completion than under static conditions *in vitro*. This agreement has not been observed with the aliphatic amides studied, viz phenylacetamide, acetamide, propionamide, which are hydrolyzed to a markedly greater extent *in vivo* than *in vitro*. There is, however, a considerable time lag in the excretion of these compounds, and it seems possible that their increased time in the body may give the enzymes responsible a better opportunity to bring about hydrolysis. This problem is still under investigation.

The isolation of 5-hydroxyanthranilic acid from hydrolyzed *o*-nitrobenzamide urine suggests that reduction of the nitro group precedes hydroxylation, since this compound was the principal hydroxylation product isolated from hydrolyzed *o*-aminobenzamide urine (Bray *et al* 1948b). Indirect support is provided by our failure to detect a nitro-hydroxybenzoic acid and by the colour reactions which suggested the presence of 3-hydroxyanthranilic acid. On purely chemical grounds the compound to be expected if hydroxylation preceded reduction would be 4-hydroxyanthranilic acid. The extent of conjugation with sulphate is, however, greater with *o*-nitrobenzamide (48%) than with *o*-aminobenzamide (about 30%). It is unfortunate

that it was not possible to obtain a reliable estimate of the amino compounds formed from *o*-nitrobenzamide. The attempts at diazo estimations suggested the presence of hydroxylamino compounds in very small amounts, although no compounds which might have been formed through hydroxylamino compounds, e.g. azoxy, azo, or hydrazo compounds, could be detected. The available evidence, therefore, suggests that the sequence of reactions is



Whilst the absence of sulphate conjugation with *o*-nitro- and *o*-amino-benzoic acids suggests that reduction and hydroxylation precede hydrolysis of the carbamyl group, there is no clear evidence as to whether the reduced compounds were excreted in the form of acids or amides. Very small amounts of *o*-nitrobenzoic acid were isolated from unhydrolyzed urines, but all the reduction products were obtained from hydrolyzed urines. The fact that the extent of sulphate conjugation was greater than the estimated percentage hydrolysis of the amide does, however, suggest that some hydroxyanthranilamide is not hydrolyzed *in vivo*.

The isolation of *m*-acetamidobenzamide from *m*-nitrobenzamide urine and the absence of sulphate conjugation lends support to the view that the hydroxylated aminobenzoic acids or amides are produced by hydroxylation of the aminobenzoic acid or amide formed by reduction of the hydroxylamino compounds, and not by rearrangement of the hydroxylamine to an aminophenol.

SUMMARY

1 A study has been made of the metabolism of *o*-, *m*- and *p*-nitrobenzoic acids and amides in the rabbit.

2 The acids are excreted mainly unchanged, but a proportion (11–21%) is reduced to the corresponding aminobenzoic acids. Acetylation then occurs in the *m*- and *p*-isomers.

3 The amides are hydrolyzed to the corresponding acids. In the case of *p*-nitrobenzamide the hydrolysis is virtually complete, *m*-nitrobenzamide is hydrolyzed to an extent of at least 58% and *o*-nitrobenzamide at least 21%. Reduction (15–25%) and acetylation occur as with the acids.

4 *o*-Nitrobenzamide is the only compound of the six to increase excretion of ethereal sulphate, 48% of the dose being eliminated in this form. The principal hydroxylation product is 5-hydroxyanthranilic acid or amide.

5 Glycine conjugation of nitrobenzoic acids could not be detected in the rabbit.

6 The reduction of nitrobenzoic acids and amides by rabbit-liver extracts has been studied.

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Studies in Detoxication

19 THE METABOLISM OF BENZENE I (a) THE DETERMINATION OF PHENOL IN URINE WITH 2,6-DICHLOROQUINONECHLOROIMIDE (b) THE EXCRETION OF PHENOL, GLUCURONIC ACID AND ETHEREAL SULPHATE BY RABBITS RECEIVING BENZENE AND PHENOL, (c) OBSERVATIONS ON THE DETERMINATION OF CATECHOL, QUINOL AND MUONIC ACID IN URINE

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The fate of benzene in the body has been the subject of numerous investigations since 1867 when Schultzen & Naunyn (1867) discovered that benzene was converted to phenol in the animal body. Phenol, catechol, quinol and their conjugates (Munk, 1876; Schmiedeberg, 1881; Nencki & Giacomini, 1880; Baernstein, 1945), muconic acid (Jaffe, 1909; Thierfelder & Klenk, 1924; Drummond & Finar, 1938; Bernhardt & Gressley, 1941) and L-phenylmercapturic acid (Zbarsky & Young, 1943) have been detected in 'benzene urine' from various animals. Of these metabolites, phenol, catechol, muconic acid and phenylmercapturic acid have been isolated in a pure crystalline state. Quinol and conjugates such as glucuronides and ethereal sulphates have not been isolated. Dihydroxydihydrobenzene (dihydroxycyclohexadiene) is also a possible metabolite, for analogous compounds have been isolated as metabolites of naphthalene (Young, 1947; Booth & Boyland, 1947), anthracene (Boyland & Levi, 1935, 1936), and phenanthrene (Boyland & Wolf, 1948). It is, therefore, clear that the metabolism of benzene is a complex problem, and hence no balance sheet for the fate of benzene in the body has yet been drawn up. Furthermore, no mechanism has been put forward to account for the formation of the diverse oxidation products.

Of the benzene administered to an animal a large proportion may be eliminated unchanged via the lungs, and at least a third to a half may leave the body in this way (Nencki & Sieber, 1883; Lehmann, Gundermann & Kleiner, 1910).

The object of the present investigation was to determine the amount of phenol and other metabolites excreted by rabbits receiving benzene orally. A new method for the determination of phenol in urine in the presence of catechol and quinol was developed. This method depended on the steam distillation of phenol from urine at pH 6, and the determination of phenol in the distillate by means of the blue colour formed with 2,6-dichloroquinonechloroimide at pH 10. This reagent has been studied

qualitatively by Gibbs (1927), Baylis (1928) and Fearon (1944). Seudt (1941) has used it for the determination of pyridoxine.

2,6-Dichloroquinonechloroimide gives a pure blue colour with phenol but it also reacts with phenols which are not substituted in the para position. The reagent is unaffected by quinol, but with catechol it gives a violet colour. Catechol, however, is not volatile in steam, and phenol can be separated from it by a steam distillation.

METHODS

THE DETERMINATION OF PHENOL IN URINE

Phenol gives a blue colour in aqueous solutions with 2,6-dichloroquinonechloroimide at pH 8–10. Under rigidly controlled conditions the colour is proportional to the amount of phenol present and a few μg of phenol/ml of solution can be accurately estimated.

Reagents required

Standard solutions of phenol. (a) A 0.1% stock solution was prepared by dissolving 1 g pure phenol in 1 l water. Its exact phenol content was determined by the bromination method of Day & Taggard (1928). This solution is stable for 2 weeks. (b) Phenol solutions (0.01, 0.005 and 0.001%) were prepared from the stock solution as required.

2,6-Dichloroquinonechloroimide reagent. This reagent (subsequently called the phenol reagent) was prepared immediately before use. Dry finely powdered 2,6-dichloroquinonechloroimide (British Drug Houses Ltd, 0.1 g) was shaken for 10 min with distilled water (100 ml). The solution was filtered from undissolved solid and 10 ml of the filtrate used in each determination. If more or less than 0.1 g of the dichloroquinonechloroimide was used in the preparation of the phenol reagent, an unsatisfactory reagent was obtained. The method of preparation of the phenol reagent is simple but critical.

Buffer solutions. (a) *Phosphate buffers* (Sorensen), 11.9425 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (A.R.) were dissolved in 500 ml distilled water and 4.539 g KH_2PO_4 in 500 ml distilled water. From these two solutions were prepared the acid buffers, pH 5.2–6.8 (see Clarke, 1928) used

when distilling free phenol from urine without hydrolyzing conjugated phenol (b) *Alkaline buffer of Kolthoff & Vlesschouwer* (1927), the colour between phenol and the phenol reagent was developed at pH 10.15 which was obtained by mixing 75.4 ml of a solution of Na_2CO_3 (2.65 g anhydrous (A.R.) in 500 ml water) with 24.6 ml of a solution of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (9.55 g in 500 ml of water)

Acid solutions (a) 10N- H_2SO_4 for the hydrolysis of conjugated phenol, (b) 6% H_3PO_4 for titrating rabbit urine to the pH required for the distillation of free phenol

Apparatus

For the steam distillation of phenol from a given solution the apparatus shown in Fig 1 was used. A is the steam generator, i.e. a 2 l conical flask. Zinc dust is added to the water

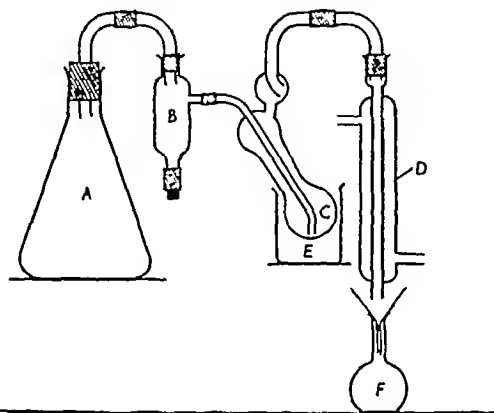


Fig 1 Apparatus for the distillation of phenol from urine (see text for description)

for smooth boiling. B is a water trap. C is a micro Kjeldahl distillation flask fitted with a bulb trap at its exit. D is a Liebig's condenser. E is a water bath (beaker) completely surrounding the end of C and the water in it is kept boiling during distillation. F is a volumetric flask used as a receiver. During the hydrolysis of conjugated phenol the condenser D is fitted vertically above C. No rubber should be exposed to the steam distillate, and the rubber bung at the upper end of the condenser D is covered with tin foil. Exposure of the distillate to rubber connexions results in a low recovery of phenol (an all glass apparatus would be ideal). Before use and between experiments the apparatus is thoroughly steamed out.

Construction of the phenol calibration curve

To a series of ten 25 ml volumetric flasks were added 1–10 ml of a standard 0.001% phenol solution ($10 \mu\text{g}/\text{ml}$). The Kolthoff-Vlesschouwer alkaline buffer pH 10.15 (5 ml) and then the phenol reagent (10 ml) were added to each flask. Finally, the volume in each flask was made up to 25 ml with distilled water and the contents mixed and allowed to stand for 1 hr when the blue colour reached a maximum. Colour measurement in a 1 cm cell was made with a Spekker photoelectric absorptiometer with an Ilford no. 607 spectrum orange filter. The curve obtained is given in Fig 2.

The effect of pH on the development of the colour was shown by repeating the above experiment using alkaline buffers of pH 9.2, 9.7 and 10.5 instead of that of pH 10.15.

At pH 9.2 maximum colour development required 3 hr, at pH 9.7 the result was similar to that at pH 10.15, at pH 10.5 the maximum colour developed in 1 hr, but it

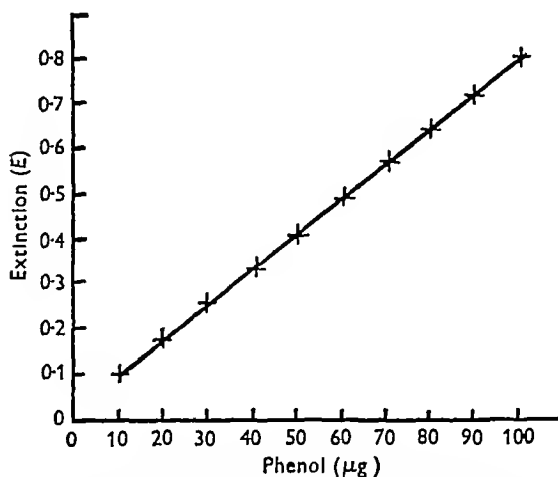


Fig 2 Standard curve for the estimation of phenol using 2,6-dichloroquinonechloroimide at pH 10.15

rapidly deteriorated, at pH 10.15 the blue colour was stable, reaching a maximum in 1 hr, and this pH was adopted in all subsequent estimations.

The recovery of phenol from pure solution by steam distillation

Mixtures of standard phenol solutions (1–5 ml) and Sorensen buffer pH 6 (5 ml) were steam distilled in the apparatus already described. Distillation was carried out at such a rate that 100 ml of liquid were distilled in 10–15 min. A suitable sample (1–10 ml) of the distillate was then buffered with the Kolthoff-Vlesschouwer alkaline buffer and the phenol reagent added as already described. The colour was measured as before. The results are given in Table 1 which shows that as little as $10 \mu\text{g}$ of phenol/ml solution can be recovered almost quantitatively by steam distillation.

Table 1 The recovery of phenol from pure solution by steam distillation

(The numbers in parentheses give the number of determinations of which the percentage recovery quoted is the average.)

Amount of phenol added	Vol of distillate (ml)	Phenol recovered (%)
1 mg in 1 ml solution	25	95.5 (8)
	50	96 (16)
	100	99 (11)
1 mg in 5 ml solution	50	96 (16)
	100	97 (16)
0.1 mg in 1 ml solution	25	92 (8)
	50	95 (4)
0.1 mg in 5 ml solution	25	91 (8)
	50	100 (4)
50 μg in 1 ml solution	25	98 (4)
50 μg in 5 ml solution	25	94 (4)

The determination of conjugated phenol in pure solution

The urines of animals receiving benzene or phenol are likely to contain phenylglucuronide and ethereal sulphate. The ethereal sulphates are readily hydrolyzed by dilute acid, but the stability of glucuronides to acid is variable (see Hanson, Mills & Williams, 1944). Menthylglucuronide, for example, is readily hydrolyzed by dilute acid, whereas o-aminophenylglucuronide is only hydrolyzed with difficulty. Phenylglucuronide appears to fall somewhere in between these two extremes. The following experiment was, therefore, designed to find out what length of time and strength of acid are required to hydrolyze phenylglucuronide completely, and whether the phenol thus set free can be recovered quantitatively by steam distillation.

A standard aqueous solution of phenyl- β -D-glucuronide (m.p. 160–162°) was prepared by dissolving 287.2 mg of the glucuronide in 100 ml water. This solution on complete hydrolysis should yield 1 mg phenol/ml. When freshly prepared these solutions give no colour with the phenol reagent at pH 10.15. However, aqueous solutions of phenylglucuronide do not keep more than a few days and should be made up fresh on each occasion.

In earlier experiments HCl was used in the hydrolysis, but since this acid is volatile in steam it was subsequently replaced by H_2SO_4 . Phenylglucuronide solution (1 ml) with 5 ml 2N or 10N- H_2SO_4 were refluxed at 100° in the micro Kjeldahl flask (C, Fig. 1) for varying times. The water in E (Fig. 1) was then replaced by ice water and the reflux condenser washed down with a few ml of water. The condenser was then put in position for distillation and the solution steam distilled. The phenol in the distillate was then determined as before. Table 2 shows that optimum recovery (95%) of phenol occurs when the glucuronide solution has been hydrolyzed for 1 hr with 10N- H_2SO_4 and these conditions were adopted for determination of conjugated phenol.

Table 2 *Recovery of phenol from phenylglucuronide solutions*

(1 ml of phenylglucuronide solution (phenol equiv = 1 mg) + 5 ml H_2SO_4 hydrolyzed and steam distilled)

Duration of hydrolysis (hr)	Strength of acid (H_2SO_4) (N)	Phenol recovered (%)
1.5	2	30
0.25	10	81.5
0.5	10	88, 89, 99, 88
1.0	10	92, 99, 91.5, 93.5, 97.5
1.5	10	90, 93.5

During the action of hot mineral acids on glucuronic acid, furfural is formed. This aldehyde can be detected (by aniline acetate) in the distillates from the hydrolysis of phenylglucuronide. Furfural is said to give a green colour with 2,6-dichloroquinonechloroimide (Seudt, 1941). There is, therefore, the possibility that furfural in the distillates may

interfere with the development of colour. It was found, however, that furfural in amounts equivalent to the phenol distilled had no effect on the development of the phenol colour. Calibration curves constructed by distilling phenol (10–100 μ g) alone and in the presence of equivalent amounts of twice distilled furfural were coincident.

The recovery of phenol added to normal rabbit urine

Normal rabbit urine is usually alkaline and has to be brought to pH 6 before added phenol can be recovered by distillation. This can be achieved by titrating to pH 6 with 6% H_3PO_4 and then adding 5 ml of a Sorensen buffer pH 6 to 5 ml of the acidified urine. It was then found that on steam distilling such a buffered urine containing added phenol the distillate had a pH of 8–9. Such distillates did not give a pure blue colour with the phenol reagent at pH 10.15, the colours obtained were dirty blue or green. The interfering substance, especially with slightly stale urines, was eventually found to be largely NH_3 , particularly in rabbit urine. The removal of ammonia, where it occurred above a critical concentration, was achieved by treating every 5 ml of urine (brought to pH 6 with 6% H_3PO_4) with 2 g of permutit (60 mesh), and filtering. A separate experiment showed that permutit did not remove added phenol from urine.

Urine containing added phenol was therefore treated as above and steam distilled, the recoveries are given in Table 3 which shows that 50–1000 μ g of phenol can be recovered by distillation almost quantitatively when added to urine.

Table 3 *Recovery of phenol added to normal rabbit urine*

Vol of urine treated (ml)	Phenol added (mg)	Vol of distillate (ml)	Phenol recovered (%)
1	1	100	98, 100
1	1	50	100, 100
5	1	100	100, 100
5	1	50	99, 101
1	0.05	25	95
5	0.05	25	93, 97, 96.5

The phenol content of normal human and rabbit urine

Rabbit urine. The recovery of added phenol from normal urine raises the question of whether normal urine contains significant amounts of phenol. Normal rabbit urine was brought to pH 6 and treated with permutit as already described above. The treated urine (5 ml) was then steam distilled. For total phenol, 5 ml of urine was hydrolyzed by heating under reflux with 5 ml 10N H_2SO_4 and steam distilled.

Table 4 shows that normal rabbit urine may contain 10–13 μ g total phenol/ml, an amount of little significance when compared with the phenol which occurs in urine after feeding benzene or phenol. The only other figures which have been given for free and conjugated phenol in rabbit urine are those of Deichmann (1943). This worker gives, free phenol, 0–0.39 mg and conjugated phenol, 1.15–10.0 mg / 24 hr.

Table 4 *Free and combined phenol in normal rabbit urine*

Rabbit no	Volume of urine (ml /24 hr)	Phenol			
		(mg /24 hr)		(mg /100 ml)	
		Free	Total	Free	Total
72	39	0 09	0 50	0 23	1 27
73	67	0 42	0 65	0 63	0 97
74	62	0 35	0 775	0 56	1 25
75	80	0 50	0 972	0 63	1 22
Sample					
1*	—	—	—	0 32	—
2*	—	—	—	0 22	—
3*	—	—	—	0 30	—
4*	—	—	—	0 26	—
5*	—	—	—	0 37	—
6*	—	—	—	0 53	—

* Mixed urines

Table 5 *Free and combined phenol in normal human urine*

Subject	Vol of fresh random sample (ml)	Phenol		
		Free (mg)	Conjugated (mg)	Conjugated (mg /100 ml)
A G	120	0	1 23	1 0
F W	230	0	1 15	0 5
J P	204	0	1 83	0 9

Human urine Since human urine is slightly acid, there is no need to titrate it with 6% H_3PO_4 as in the case of rabbit urine. To 5 ml human urine, 5 ml of Sorensen buffer pH 6 were added and the resulting mixture had a pH very nearly 6. The urine was then treated with permutit and the filtrate distilled. Total phenol was determined as for rabbit urine. The results are given in Table 5.

General methods

Glucuronic acid was determined by the method of Hanson *et al* (1944), using pure phenylglucuronide (m p 161–162°) as the standard.

Ethereal sulphate and neutral sulphur were determined gravimetrically by the Folin method.

RESULTS

Experiments with Phenol

Free and conjugated phenol Rabbits were maintained on a diet of 50 g Lever's cubes and 100 g

cabbage *per diem*. Phenol was administered by stomach tube at a dose level of 125 mg/kg in 10 ml of water in most experiments. In two experiments phenol dissolved in 0.9% sodium chloride was injected intraperitoneally at a dose level of 60 mg/kg. Urine for analysis was centrifuged and then filtered. All determinations were carried out within 1–2 hr of collecting the urine sample. The urine was analyzed for free and conjugated phenol by the method described above.

Phenol at the dose level used is excreted almost entirely in a conjugated form, traces only of free phenol being excreted (Table 6). The excretion of free phenol is more significant after injection than after oral administration. On an average 77% of the fed phenol and 62% of the injected phenol are excreted as total phenol. Furthermore, the phenol was completely excreted within 24 hr of its administration.

Table 6 *The excretion of free and conjugated phenol by rabbits receiving phenol orally and by injection*

Rabbit no	Wt (kg)	Dose (mg /kg)	Method of administration	Phenol excreted			
				Free		Total	
				(mg)	(% of dose)	(mg)	(% of dose)
6	2 85	125	Oral	0	0	281 6	79
9	2 65	125	"	0	0	232 5	70
10	2 95	125	"	Trace	0	302 0	81 9
16	2 1	125	"	Trace	0	170 6	65
31	2 45	125	"	Trace	0	236 1	77 1
73	2 5	60	Injection	5	3 3	84 3	56 2
75	2 6	60	"	4 2	2 4	98 0	65 3

Glucuronic acid excretion The results (Table 7) show that at doses of 125 and 250 mg/kg about 70 % of the administered phenol is excreted as a glucuronide (see Fig 3) The figures for the dose of 50 mg/kg are unreliable, for at these low doses, the variation in normal glucuronic acid excretion may cause significant errors

Experiments with benzene

Free and conjugated phenol Rabbits were maintained on a constant diet of 50 g Lever's cubes and 100 g cabbage *per diem* Benzene was administered by stomach tube at a dose level of 500 mg/kg with 10 ml water The urine excreted was analyzed daily

Table 7 The excretion of conjugated glucuronic acid in the rabbit after orally administered phenol

Rabbit no	Wt (kg)	Dose (mg/kg)	'Extra' glucuronic acid excreted (mg)	Phenol equivalent to 'extra' glucuronic acid (% of dose)
66	2.82	50	314.0	107.9
68	2.8	50	271.4	93.2
72	2.75	50	325.6	114.7
75	2.62	50	264.6	96.7
78	2.5	125	548.5	85.0
82	2.45	125	494.2	78.3
83	2.65	125	308.0	58.2
66	2.77	250	1094.5	77.1
68	2.8	250	1216.7	83.6
72	2.7	250	875.0	62.8
75	2.6	250	706.8	52.6

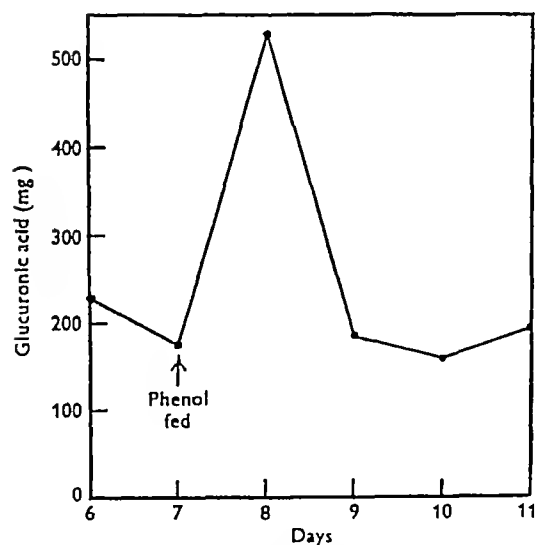


Fig 3 Glucuronic acid excretion by rabbit no 66 which had received 141 mg of phenol orally on the 7th day of the experiment

Isolation of phenylglucuronide Five large rabbits were each given 0.9 g of phenol (total fed, 4.5 g) with water Their urine, collected during 18 hr, was acidified with a little glacial acetic acid and treated with 0.2 vol of saturated normal lead acetate solution The solution was filtered and the filtrate neutralized with NH_4OH An excess of saturated basic lead acetate solution was now added and the precipitate was filtered at the pump and washed with water It was suspended in water and its lead removed with H_2S After removal of PbS the filtrate was concentrated *in vacuo* at 40–50° until it began to crystallize The whole was allowed to crystallize at 0° and the phenylglucuronide (4–6 g) filtered off It was recrystallized from benzene containing a little ethanol and it formed needles m.p. 161–162° sintering slightly at 110°

for free and conjugated phenol by the method already described (see Table 8) Most of the phenol formed was excreted within 24 hr of feeding the benzene (see Fig 4) and furthermore very little occurred in the free state

Excretion of conjugated glucuronic acid The output of glucuronic acid after feeding benzene is given in Table 9 Fig 5 illustrates a single experiment These results show that on an average about 11 % of the dose of benzene is excreted as a glucuronide, assuming that one molecule of benzene gives rise to one of 'extra' glucuronic acid

Excretion of ethereal sulphate and neutral sulphur The results for these experiments are given in Table 10 In two of these experiments the benzene was fed dissolved in a vegetable oil, and in the others with 10 ml of water An average of 9.5 % of the benzene fed is excreted as an ethereal sulphate The main bulk of the 'extra' ethereal sulphate is excreted during the first 24 hr after feeding (see Fig 5), although in one or two cases appreciable amounts were excreted in the second 24 hr

In the case of the neutral-sulphur output, the daily variation of the normal value in the rabbit was such that it was difficult to reach a definite conclusion We are, however, of the opinion that if any benzene is excreted by the rabbit as a mercapturic acid then the amount is small, and cannot be detected by neutral-sulphur determinations Witter (1945) has concluded that the rabbit probably does not excrete phenylmercapturic acid, but Zbarsky & Young (1943) have isolated this conjugate from rat urines in yields up to 0.37 % of the dose of benzene Such amounts could not be detected by neutral-sulphur determinations

Table 8 *The excretion of phenol by the rabbit after oral administration of benzene (dose 500 mg/kg)*

Rabbit no	Wt (kg)	Dose (g)	Phenol excreted			
			Free (mg)	Total (mg)	Free (% of dose)	Total (% of dose)
7	2.65	1.325	0	271.0	0	17.0
36	2.15	1.075	0	118.2	0	9.1
41	2.0	1.000	0	117.7	0	9.7
44	1.85	0.925	0	65.3	0	5.9
72	2.65	1.300	24.6	112.9	1.8	7.2
74	2.6	1.300	6.2	98.0	0.4	6.3

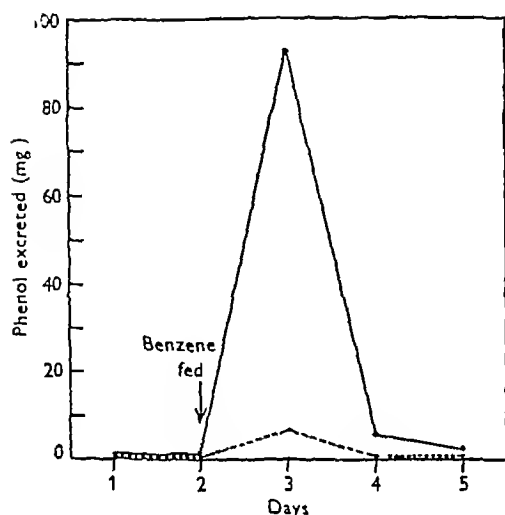


Fig. 4. The excretion of free (broken line) and total (continuous line) phenol by rabbit no. 74 after an oral dose of 1.3 g benzene

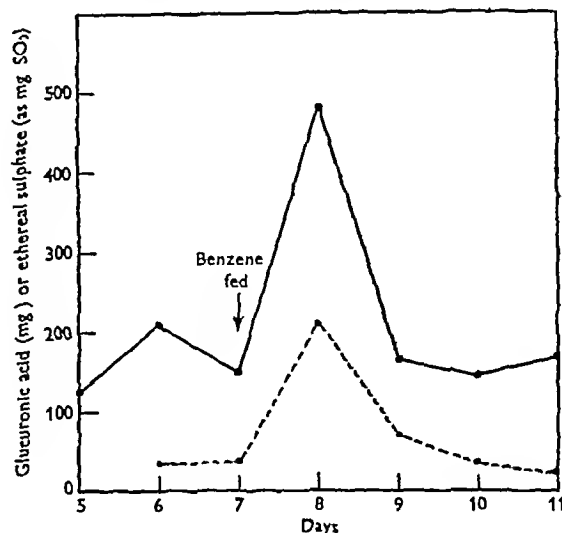


Fig. 5. Glucuronic acid excretion by rabbit no. 84 (continuous line) and ethereal sulphate excretion by rabbit no. 9 (broken line), after oral doses of benzene. Doses: rabbit no. 84, 1.3 g (500 mg/kg), rabbit no. 9, 2.85 g (10 g/kg)

Table 9 *Excretion of conjugated glucuronic acid by rabbits receiving orally 500 mg of benzene/kg*

Rabbit no	Wt (kg)	Dose (g)	Extra glucuronic acid (mg)	Extra glucuronic acid (% of dose)
73	2.65	1.325	139.5	4.2
79	2.45	1.235	149.0	4.8
74	2.5	1.25	182.6	5.9
79	2.55	1.275	260.5	7.9
84	2.65	1.325	268.8	8.15
83	2.57	1.3	279.8	8.6
84	2.6	1.3	309.7	9.5
79	2.45	1.225	418.5	13.7
82	2.5	1.275	556.6	17.5
84	2.57	1.31	681.4	20.9
83	2.55	1.31	739.7	22.7

Table 10 *The excretion of ethereal sulphate and neutral sulphur by rabbits receiving benzene orally (dose 500 mg/kg)*

Rabbit no	Wt (kg)	Dose (g)	Extra ethereal sulphate		Neutral sulphur (% of dose)
			(mg SO ₂)	(% of dose)	
7	2.5	1.3*	78.2	5.85	0
6	2.8	1.4*	113.8	7.9	0
6	2.7	1.35*	110.9	8.0	0
9†	2.85	2.694*	231.3	8.4	0
10†	2.9	2.893*	250.4	8.4	0
6	2.65	1.31*	140.2	10.4	0
6	2.6	1.30†	142.8	10.7	0
7	2.6	1.30†	224.2	16.8	0

* Benzene fed with water

† Dose of 1 g/kg

‡ Benzene fed dissolved in oil

Observations on catechol and quinol

The only previous attempt to estimate catechol and quinol in urine appears to be that of Baernstein (1945). In this, the phenols were extracted from neutralized acid-hydrolyzed benzene urines with ether, and the catechol was separated from phenol and quinol as a lead salt. We attempted unsuccessfully to find specific reagents for catechol and quinol in order to avoid lead separation. Although we devised methods for the determination of catechol and quinol added to normal urine, these methods gave erratic results when applied to benzene urines owing to the presence of interfering substances, one of which we identified as hydroxyquinol. It is, therefore, proposed to mention our experiments only briefly.

Determination of catechol in pure solution Catechol can be readily estimated in pure solution with 2,6-dichloroquinonechloroimide. With this reagent catechol gives at an optimum pH of 6.6–6.8 a blue-purple colour which is proportional to the catechol present. Quinol does not interfere, but phenol and *o*- and *m*-cresols do. A calibration curve was prepared by mixing, in 20 ml flasks, acetate buffer pH 6.7 (5 ml) and a solution of 2,6-dichloroquinonechloroimide (3 ml) prepared as for phenol estimations, with a series of 1–10 ml of a fresh 0.002% catechol solution and making up to 20 ml with water. The colours produced 1 hr after mixing were read in a Spekker absorptiometer with an Ilford no. 606 spectrum yellow filter. A reproducible straight line calibration curve was obtained.

Catechol in quantities of 0.1–1.0 mg/ml can be recovered from aqueous solution by ether extraction for 4 hr to the extent of 80% of that added.

Determination of quinol in pure solution The estimation of quinol was based on the method of Oglesby, Sterner & Anderson (1947) for the determination of *p*-benzoquinone in air. The standard curve was obtained by mixing in 20 ml flasks a series of 1–10 ml of a 0.002% quinol solution with 1 ml of 0.5% phloroglucinol and then adding water to within 3 ml of the mark. 0.1N-KOH (1 ml) was added and then water to 20 ml. The mixed solutions were kept for 1 hr and then the red colour was measured in the absorptiometer using an Ilford no. 604 spectrum green filter. A reproducible straight line calibration curve was obtained.

By this method quinol can be estimated in the presence of phenol, *o*-, *m*- and *p*-cresols and furfural, but catechol interferes. Quinol (0.05% solutions) can be recovered by ether extraction for 4 hr from aqueous solution to the extent of 90%.

Neither of these methods for catechol and quinol is as sensitive as the method used for phenol.

Determination of added catechol and quinol in urine Normal rabbit urine (5 ml) containing 10 mg each of catechol and quinol were acidified with 5 drops of 2N H_2SO_4 and then extracted continuously for 4 hr with ether. Acetate buffer pH 6.7 (10 ml) was added to the extract and the ether was removed from the mixture by suction at room

temperature. The resulting solution was then neutralized with solid $NaHCO_3$, filtered and made up to 25 ml with the acetate buffer pH 6.7. A 5 ml sample of the solution was then placed in a fine sintered glass filter (Pyrex SF2A/4) and mixed with 5 ml buffer pH 6.7 and 5 ml 0.1M lead acetate (normal) solution. Catechol is thus precipitated as a lead salt (precipitate A). The glass filter was then subjected to suction, the filtrate being sucked into a test tube containing 5 ml of 2% sodium oxalate which precipitated the excess lead in the filtrate. The excess lead was removed by filtration through asbestos in a Gooch crucible and the filtrate made up to 50 ml with water. The quinol in a sample of this solution was then determined by the phloroglucinol KOH method described above. Glacial acetic acid (3 ml) was now added to the lead catecholate (precipitate A) in the sintered glass filter. When the lead salt had dissolved, the resulting solution was sucked into a test tube containing 5 ml of 2% sodium oxalate. The glass filter was carefully washed with 10 ml water which was also sucked into the test tube. The mixture was then filtered through the same glass filter to remove lead oxalate, and the final clear filtrate, after neutralization with solid $NaHCO_3$, was made up to 50 ml with water. Catechol was determined in 5 ml samples of this solution as described above for catechol in pure solution. The recoveries of both catechol and quinol by this method were 80–90% of the amounts present. Such recoveries were also obtained when phenol was present, for phenol appears in the quinol fraction during the lead separation.

The recovery of catechol and quinol was also tested under hydrolysis conditions. Urine (5 ml) containing the added phenols was heated on the water bath for 1 hr under reflux with 10N- H_2SO_4 (5 ml). The mixture was cooled and extracted with ether for 4 hr. Catechol and quinol in the extract were then determined as in the preceding paragraph. Under these conditions 80% of the added phenols were recovered.

The above method was now applied to urines of rabbits receiving benzene or phenol orally. With benzene urines erratic results were obtained due apparently to the other metabolites in such urines. The results were more erratic for catechol than for quinol, for we found that benzene urine contained hydroxyquinol which was precipitated with catechol during the lead separation. Our results with benzene are therefore not worth quoting.

Some results with administered phenol are given in Tables 11 and 12. It should be noted that no free catechol or quinol was excreted. These tables show that the main oxidation product of phenol is quinol. After a single dose of phenol, quinol is excreted, but not catechol. With continuous feeding of phenol about 5% of the dose appears as conjugated quinol, with traces only of catechol. These results confirm the earlier observations of Baumann & Preusse (1879), who isolated quinol from the hydrolyzed urine of a dog whose skin had been painted with phenol twice a day for 6 days, and from the urine of a dog poisoned with phenol, catechol was only detected in small amounts. Baumann & Preusse also detected quinol in the urine of dogs receiving

Table 11 *The excretion of combined catechol and quinol by a rabbit receiving daily doses (250 mg /kg) of phenol*

Day	Dose (g)	Volume of urine (ml)	Catechol		Quinol	
			(% of the day's dose)	(% of the total fed)	(% of the day's dose)	(% of the total fed)
1	0.727	60	0	0	2.3	2.3
2	0.721	138	0	0	7.6	4.95
3	0.717	84	Traces	0	Present*	?
4	0.710	115	0	0	5.1	5.0†
5	0.712	140	0	0	4.8	4.95†

* Result unreliable

† Excluding day 3

Table 12 *The excretion of catechol and quinol by a rabbit receiving a single oral dose (200 mg /kg) of phenol*

Day	Dose (g)	Percentage of the dose excreted as			
		Catechol		Quinol	
		Free	Combined	Free	Combined
1	0.62	0	0	0	1.0
2	Nil	0	0	0	0
3	Nil	0	0	0	Trace

doses of potassium phenylsulphate, but in lesser amounts than after phenol, the phenylsulphate being more rapidly excreted than phenol. It should also be noted (see Tables 11 and 12) that continuous dosing with phenol gives rise to the excretion of larger amounts of quinol than does a single dose.

Muconic acid

If the ring of benzene undergoes fission in the body to muconic acid it should give rise to the *cis-cis* form, but the isomer which has been isolated from benzene urines in the past has always been the *trans-trans* form (see Williams, 1947). The amount of muconic acid formed from benzene is small and uncertain so we attempted to find a method for estimating this acid in urine. The occurrence of a conjugated double bond system in the acid immediately suggested that it might be estimated spectroscopically. We have, therefore, examined the ultraviolet absorption spectra of the two isomers (see Fig. 6).

cis-cis Muconic acid, m.p. 175°, was prepared according to Böeseken & Kerkhoven (1932). Its absorption spectrum has not been previously recorded. In ethanol it shows one absorption band with λ_{\max} 258.5 m μ and ϵ_{\max} 19,880.

trans-trans Muconic acid, m.p. 296°, was prepared according to Ingold (1921). Its ultraviolet absorption spectrum in ethanol has been previously recorded by Hausser, Kuhn, Smakula & Deutsch (1935) who give λ_{\max} at 261–263 m μ , but ϵ_{\max} was not quoted. In ethanol we found one band with λ_{\max} 259 m μ and ϵ_{\max} 27,100 and in 2% NaOH the band was at 261 m μ and ϵ_{\max} 24,600. Thus both forms of muconic acid show maximum absorption at the same wave length, but the *trans-trans* isomer absorbs more strongly than the *cis-cis* isomer (cf. *cis-* and *trans-cinnamic* acids, Smakula & Wassermann, 1938). It is possible that during isolation from urine the *cis-cis* acid may be converted to the

trans-trans acid (cf. Drummond & Finar, 1938). We have investigated the possibility that this change may be brought about by heat, our criterion of change being alteration in the extinction at 259 m μ . We found, however, that the *cis-cis* acid in ethanolic solution (0.025%) was not converted to the *trans-trans* acid by heat alone.

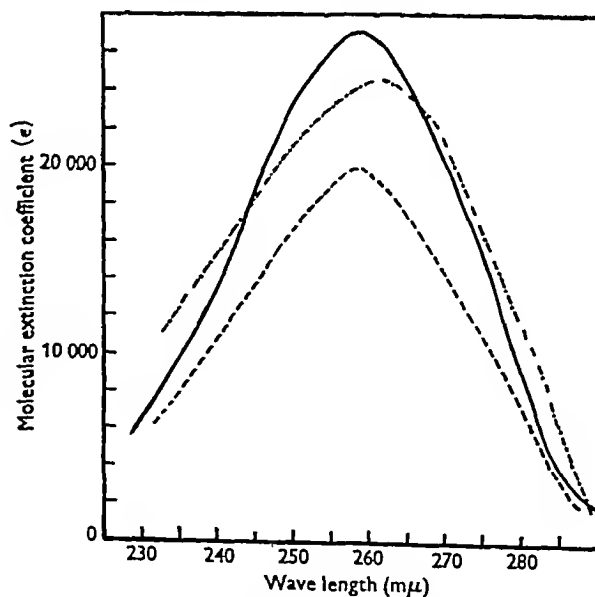


Fig. 6 Spectral absorption of *cis-cis*- and *trans-trans*-muconic acids — *trans-trans*-Muconic acid in ethanol, λ_{\max} 259 m μ , ϵ_{\max} 27,100, - - *trans-trans* muconic acid in 2% NaOH, λ_{\max} 261 m μ , ϵ_{\max} 24,600, - - - *cis-cis* muconic acid in ethanol, λ_{\max} 258.5 m μ , ϵ_{\max} 19,880.

The determination of *trans-trans* muconic acid in pure solution and in urine. *trans-trans* Muconic acid is adsorbed by an alumina column from aqueous solution and it can be eluted by 2% NaOH. The amount of the acid in the eluate

can be determined spectrographically. For example, 2.5 mg muconic acid in 200 ml water were completely adsorbed by a column of alumina (Savory and Moore Ltd) 20×1.25 cm. After washing the column with 100 ml water, the muconic acid was completely eluted by 50 ml 2% NaOH, and the muconic acid content of the eluate was determined spectrographically. In 8 experiments using 2.5 mg of the acid in 200 ml water the following recoveries were obtained, 100, 86, 96, 96, 100, 109, 92 and 100%. With shorter columns the muconic acid was not completely adsorbed. The same experiment was repeated with muconic acid added to normal rabbit urine at varying pH, but on eluting the column with 2% NaOH it was found that some constituent of the urine, which absorbed all light of wave lengths less than $300 m\mu$, was also eluted. Although much time was spent on this problem, it finally had to be abandoned.

DISCUSSION

In Table 13 the results quoted in preceding tables have been averaged and summarized. With benzene there is a considerable scatter in the glucuronide figures which vary from 4–22%. The figures for ethereal sulphate (5–10.7%, with one extreme of 16.8%) and phenol (6.3–9.7%, with one extreme of 17%) are more consistent. This scatter may in part be due to the volatility of benzene, for there is little doubt that a large and variable amount of the benzene fed escapes unchanged through the lungs. Although we have not yet investigated the elimination of unchanged benzene we suspect that the amount thus eliminated may be as high as 80% of the dose. The scatter in the glucuronide figures is also partly due to the quantitative method being less accurate than the methods for ethereal sulphate and phenol. We can, however, use the averaged figures of Table 13 for the purposes of discussion.

total phenols that 25% of injected benzene was oxidized to phenols in the rabbit. We have also found that the phenols excreted are almost entirely conjugated. Small amounts of free phenol but no free catechol, quinol and hydroxyquinol were found. Concerning the amounts of the latter phenols excreted it appears that together they amount to about 11% of the dose of benzene, but we were unable to estimate each phenol separately for reasons already mentioned. The qualitative results of the following paper (Porteous & Williams, 1949), however, indicate that catechol and quinol are probably excreted in roughly equal amounts, whereas hydroxyquinol excretion is about a quarter that of catechol. A very rough assessment of the amounts of each phenol excreted would be phenol 10, catechol 4–5, quinol 4–5 and hydroxyquinol about 1% of the dose of benzene.

With reference to phenol itself it is clear that its main metabolites are phenylglucuronide and phenylsulphuric acid which together account for 75% of the phenol fed. Furthermore, we found that only traces of free phenol were excreted, a result which is contrary to that found by Deichmann (1944), who reports that half of the phenol excreted in the urine of rabbits receiving phenol orally is in the free state. That our results are correct is supported by the fact that the total conjugation is 89% which in experiments of this type represents an almost quantitative recovery of the phenol fed. The difference between the total conjugation and total phenol amounts to 14% (see Table 13). This figure, which is necessarily only approximate, indicates that phenol is converted to a small extent into other phenols which, according to the early work of Baumann & Preusse (1879), are

Table 13 *The excretion of metabolites by rabbits receiving benzene or phenol orally (averaged results)*

Compound fed	Dose (mg/kg)	Percentage of dose excreted as				
		Ethereal sulphate	Glucuronide	Total phenol	Total conjugates	Other phenols (i.e. total conjugates – total phenols)
Benzene	500	9.5	11.2	9.2	20.7	11.5*
Phenol	125–250	19†	70	75	89	14†

* Catechol, quinol and hydroxyquinol.

† Value quoted from Williams (1938)

‡ Mainly quinol

This table shows that about 20–21% of the benzene fed is excreted as conjugated sulphates and glucuronides, the ratio glucuronide/ethereal sulphate being roughly unity. About one half of these conjugates contains phenol and the other half contains catechol, quinol and hydroxyquinol (Porteous & Williams, 1949). Thus about 20–21% of the administered benzene is oxidized to phenols, a figure which agrees with the results of Braunstein, Parschun & Chalisowa (1931), who found by direct estimation of

mainly quinol with small amounts of catechol. In fact Baumann & Preusse isolated quinol from the urine of a dog which had had phenol rubbed into its skin. Our results on the determination of catechol and quinol in phenol urine indicate that at least 5% of the phenol fed is excreted as quinol conjugates. Whether or not phenol is oxidized to catechol in the higher animals has yet to be proved. In certain phenol-utilizing micro-organisms, however, phenol is oxidized to catechol (Evans, 1947).

SUMMARY

1 A quantitative study has been made of the excretion of phenol, glucuronic acid and ethereal sulphate by rabbits receiving oral doses of benzene or of phenol

2 A new colorimetric method for the estimation of free and combined phenol in urine has been studied. This method is based on steam distillation of phenol from urine and determination of phenol in the distillate by means of the blue colour with 2,6-dichloro-quinonechloroimide under specified conditions

3 Normal human urine contains about 5–10 mg of total phenol/l in a conjugated form, whereas rabbit urine contains 2–6 mg free and 10–13 mg total phenol/l

4 The results indicate that about 21% of administered benzene is excreted as phenols, 9.2% as

phenol and 11.5% as other phenols, 9.5% of the benzene fed is excreted as ethereal sulphates and 11.2% as glucuronides. Only traces of free phenol are excreted

5 When phenol is fed to rabbits it is largely excreted as phenylglucuronide and phenylsulphuric acid, but some is oxidized to quinol which is excreted conjugated. Only small amounts of free phenol are excreted

6 Attempts to estimate catechol and quinol in benzene urine were unsuccessful because of interference by hydroxyquinol, a metabolite of benzene.

7 The absorption spectra of *cis-cis*- and *trans-trans* muconic acid are recorded. These acids can be estimated in pure solution spectrographically, but the method fails with urine

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Studies in Detoxication

20 THE METABOLISM OF BENZENE II THE ISOLATION OF PHENOL, CATECHOL, QUINOL AND HYDROXYQUINOL FROM THE ETHEREAL SULPHATE FRACTION OF THE URINE OF RABBITS RECEIVING BENZENE ORALLY

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In the preceding paper it was shown that the average sum of the glucuronic acid and etheral sulphate conjugations of rabbits receiving benzene orally was about 20 %, and it was assumed that the conjugates excreted were those of phenolic substances. Of these phenols about one half was shown quantitatively to be phenol whereas the other half was probably a mixture of other phenols such as catechol and quinol. In the present paper it will be proved by actual isolation that the urine of benzene-fed rabbits contains four phenols, namely phenol, catechol, quinol and hydroxyquinol (1,2,4-trihydroxybenzene). Phenol and catechol have been isolated from 'benzene urines' by earlier workers (Munk, 1876, Nencki & Giacomini, 1880), but we report the isolation of quinol and hydroxyquinol for the first time. We also looked for pyrogallol and dihydroxydihydrobenzene, but found no evidence that they are metabolites of benzene.

EXPERIMENTAL

The colour reactions used for the detection of phenols

During the extraction and separation of phenols from benzene urine, the nature of the phenols in the fractions separated was ascertained by the following colour tests.

The ferric chloride test. To 2–3 ml of the neutral or faintly acid test solution, 1 or 2 drops of a 2 % aqueous FeCl_3 solution were added. The colour was noted and then the solution was made progressively more alkaline, first with solid NaHCO_3 , and finally with 1 drop of 2N NaOH . Any change in colour was noted during the progressive alkalization of the solution. The colours given by the relevant phenols are given in Table 1.

The most intense colours are given by catechol and hydroxyquinol, and it is not possible to distinguish between catechol, hydroxyquinol and pyrogallol. In mixtures of catechol and hydroxyquinol, such as those encountered in extracts of benzene urine, it is not possible to say by this test whether or not hydroxyquinol is present.

The 2,6-dichloroquinonechloroimide test. To 2–3 ml of the test solution, a few small crystals of 2,6-dichloroquinonechloroimide (British Drug Houses Ltd.) were added and the solution well agitated. Any colour was noted. The pH of the solution was now gradually raised to 8 by small additions of solid NaHCO_3 , the solution being agitated continuously. Time was given for any colour to develop. The pH was further raised to 9.2 by adding borax solution and the colours again noted. Finally the pH was raised to 10 with a Na_2CO_3 borax solution. The results of these tests are recorded in Table 2; quinol is not included for it does not give a colour with the reagent. In mixtures of the relevant phenols, hydroxyquinol was found to give the dominant colour, and it was found easy to detect it when large amounts of catechol were present. The blue colour given by phenol develops slowly at pH values less than 10 and hydroxyquinol can easily be detected in the presence of phenol. The present test was found to be very useful for detecting small amounts of hydroxyquinol in the presence of catechol and phenol.

The sulphuric acid test (cf. Barth & Schreder, 1884). To a few crystals of the dry phenol 4 or 5 drops of cold conc. H_2SO_4 were added and the colour observed. The mixture was then warmed for a few seconds on the water bath and the colours observed. Table 3 shows that the H_2SO_4 test is almost specific for hydroxyquinol if the other phenols present are those included in Table 3. With dried evaporated ether extracts from urine, H_2SO_4 may sometimes give brownish colours due to charring. However, with experience hydroxyquinol can easily be detected in extracts by this and the previous test.

Table 1 *The ferric chloride reaction of certain phenols*

'Phenol'	Colour appearing in			
	Neutral solution	NaHCO_3	Trace of NaOH	1 drop of 2N NaOH
Phenol	Pale violet	Colourless	Ppt $\text{Fe}(\text{OH})_3$	—
Quinol	Green flash, then yellow	Colour fades	Ppt $\text{Fe}(\text{OH})_3$	—
Resoreinol	Pale violet	Yellow	Ppt $\text{Fe}(\text{OH})_3$	—
Phloroglucinol	Intense violet	Ppt $\text{Fe}(\text{OH})_3$	—	—
Hydroxyquinol	Dark brown	Blue	Purple	Red-purple to red
Pyrogallol	Transient green, then red brown	Brown	Purple	Brown purple
Catechol	Dark green	Intense violet	Purple	Red purple

Table 2 The colour reactions of certain phenols with 2,6-dichloroquinonechloroimide and the effect of pH

Compound	Colour produced at pH				Comment
	6.5-7.0	8.0	9.2	10.0	
Phenol	Blue	Blue	Blue	Blue	Intensifying as pH rises
Catechol	Blue purple	Blue purple	Blue purple	Red purple	Colour unstable with rising pH
Resorcinol	Magenta	Red	Crimson	Dark red with ppt	—
Hydroxyquinol	Red	Red	Intense red	Very intense red tending to fade to brown	Colour intensifies with rising pH and stable up to pH 10
Phloroglucinol	Orange red	Orange	Yellow	Fades to brown	—
Pyrogallol	Yellow	Yellow brown	Brown	Cherry red	—

As hydroxyquinol was actually isolated as its tri-*p* toluenesulphonate, it is included in Table 3. This ester gives an intense green colour on heating with conc. H_2SO_4 . The *p* toluenesulphonates of phenol, catechol and quinol give only faint yellowish or reddish brown colours in this test.

Table 3 The colour reactions of certain phenols with concentrated sulphuric acid

'Phenol'	Colour obtained with conc. H_2SO_4	
	In the cold	On warming
Phenol	Colourless solution	Colourless solution
Catechol	Colourless solution	Colourless solution
Resorcinol	Pink	Faint orange
Quinol	Colourless solution	Colourless solution
Hydroxyquinol	Pink	Intense red
Phloroglucinol	Colourless solution	Colourless solution
Pyrogallol	Colourless solution	Colourless solution
Hydroxyquinol tri- <i>p</i> toluene sulphonate	Colourless solution	Intense green*

* Intensifies on stronger heating, the *p* toluenesulphonates of phenol, catechol and quinol do not give a characteristic colour.

Table 4 The melting points of the benzoates and *p*-toluenesulphonates of certain phenols

Compound	Melting point of	
	Benzoate	<i>p</i> Toluene sulphonate
<i>p</i> Cresol*	71°†	69°†
Phenol	68°†	95°†
Catechol	84° (di)†	162-3° (di-)‡
Quinol	199° (di)†	159° (di)§
Hydroxyquinol	120° (tri)	105-6° (tri-)‡
Pyrogallol	89-90° (tri-)†	140° (tri)‡

* *p* Cresols included as a common phenol of normal urine.

† Wild (1947).

‡ This paper.

§ Hopkin & Williams Ltd (1944).

|| Thiele & Jaeger (1901).

Preparation of reference compounds

For the separation and identification of the phenols of benzene urine, it was necessary to prepare and study the solubilities of a number of phenolic esters. The benzoates and *p* toluenesulphonates were found to be the most useful

and easily prepared derivatives. A summary of the melting points of reference compounds is given in Table 4.

The *p* toluenesulphonates of catechol, hydroxyquinol and pyrogallol are new compounds. They were prepared by dissolving the phenol (1 g) in 10% NaOH (10 ml) and adding 10 ml acetone containing the calculated amount of *p* toluenesulphonyl chloride. The mixture was shaken in a stoppered tube for 10-20 min. The product was poured into water, and the solid formed was recrystallized from aqueous or absolute ethanol. In the case of hydroxyquinol (m.p. 138°, prepared according to Vliet, 1925 and Healy & Robinson, 1934) good yields of the ester were obtained only if Na_2CO_3 were used instead of NaOH.

Catechol di-*p* toluenesulphonate formed small colourless needles, m.p. 162-163°, from ethanol (Found C, 57.4, H, 4.3, S, 15.1. $\text{C}_{20}\text{H}_{16}\text{O}_6\text{S}_2$ requires C, 57.4, H, 4.3, S, 15.3%).

Hydroxyquinol tri-*p* toluenesulphonate formed rectangular plates, m.p. 105-106°, from ethanol (Found C, 55.7, H, 4.3, S, 16.2. $\text{C}_{27}\text{H}_{24}\text{O}_9\text{S}_3$ requires C, 55.1, H, 4.1, S, 16.3%).

Pyrogallol tri-*p* toluenesulphonate formed irregular plates, m.p. 137-140°, from ethanol (Found C, 54.9, H, 4.3, S, 16.5. $\text{C}_{27}\text{H}_{24}\text{O}_9\text{S}_3$ requires C, 55.1, H, 4.1, S, 16.3%).

The examination of benzene urine for phenols

On two successive days, 10 rabbits, on a diet of 50 g Lever's cubes and 100 g cabbage/day, were given by stomach tube 2 ml benzene each, i.e. a total of 40 ml. or 32 g. The urines were collected for 4 days (hereafter referred to as U_1 , U_2 , U_3 and U_4) after the first dose. The daily volumes of urine obtained were 800, 1220, 800 and 1000 ml respectively. All samples had a pH of 8, were dark in colour, non-reducing and gave no definite colour with FeCl_3 . Each urine was centrifuged to remove any solid material. Peroxide free ether was used in all extractions.

Isolation of free phenol. Only the urine of the first day U_1 was examined for free phenol. This untreated urine was extracted for 3.5 hr with ether in a continuous extractor. The extract was colourless and colour tests indicated that only phenol was present. The ether was evaporated and the residue was dissolved in 10 ml of 10% NaOH. To this 2 g *p* toluenesulphonyl chloride in 5 ml acetone were added. The mixture was shaken for 0.5 hr and kept overnight. It was then poured into 50 ml water and the precipitate produced was collected and recrystallized (charcoal) from hot absolute ethanol. The product (plates, 50 mg) had m.p.

90-91° and was identified as phenol *p* toluenesulphonate, mixed m p 90° (Found C, 62.8, H, 4.7, S, 12.6 Calc for $C_{13}H_{12}O_3S$ C, 62.4, H, 4.9, S, 13.1 %)

Partial hydrolysis of the urine Each day's urine was treated with enough conc HCl to give it an acidity of 1N and then it was heated to 90° for 0.5 hr. By this process ethereal sulphates but not glucuronides were hydrolyzed, for, as shown in the preceding paper (Porteous & Williams, 1949), much stronger acid and longer heating is necessary to hydrolyze phenylglucuronide.

After hydrolysis U_1 and U_2 were extracted with ether for 37 hr, 10 fractions being collected. Colour tests on these indicated that earlier fractions contained mainly phenol and catechol with small amounts of quinol, whereas the later fractions contained mainly quinol with small amounts of catechol and phenol. Only traces of hydroxyquinol were found in U_1 and U_2 . The combined ether extracts were dried overnight with anhydrous Na_2SO_4 and then evaporated by suction at room temperature. To minimize the loss of volatile phenols heating was avoided during evaporation of the ether. In this way an oily residue was obtained.

Separation of phenol and quinol from catechol and hydroxyquinol Phenol and quinol were now separated from catechol and hydroxyquinol by lead precipitation. The oily mixture was taken up in the minimum quantity of boiling water, treated with charcoal and filtered. On cooling, hippuric acid often crystallized out at this stage and was removed by filtration. The filtrate was made faintly alkaline with $NaHCO_3$ and an excess of saturated basic lead acetate solution added. The precipitate which formed was filtered off and washed with water. This precipitate (A) contained the catechol and hydroxyquinol and the filtrate (B) from it, the phenol and quinol.

Isolation of catechol The precipitate (A) was suspended in water and thoroughly gassed with H_2S . The PbS was filtered off and the filtrate, after treatment with charcoal to remove colloidal sulphur, was acidified with a little HCl. It was then extracted continuously for 20 hr with ether. The extract was evaporated and the residue benzoylated with 2.5N-NaOH and benzoyl chloride. The mixture was poured into water and the alkaline solution allowed to stand 24 hr. The precipitate was filtered, washed with water and dried (yield 550 mg, * m p 60-67°). It was then dissolved in 100 ml hot ethanol and filtered. On standing overnight a small crop (20 mg) of needles, m p 190°, appeared. This was recrystallized from methanol and identified as quinol dibenzoate, m p and mixed m p 194° (Found C, 75.5, H, 4.5 Calc. for $C_{13}H_{10}O_3$ C, 75.5, H, 4.4 %). The filtrate from this small fraction was diluted with a little water and evaporated to two thirds its volume. On cooling, 520 mg of crystalline material m p 80-95° were obtained. The above crystallization procedure was repeated and a small amount of quinol dibenzoate, m p 190°, and 500 mg of catechol dibenzoate were obtained. The catechol derivative after two recrystallizations from methanol had m p 80°, mixed m p 81-82° (Found C, 75.3, H, 4.5 Calc for $C_{13}H_{10}O_3$ C, 75.5, H, 4.4 %).

Isolation of phenol and quinol The filtrate B (see above) was thoroughly treated with H_2S to remove excess of lead. The solution was filtered, treated with charcoal to remove colloidal sulphur, and the clear solution acidified with a

little HCl. It was then extracted for 15 hr with ether. The oily residue, after removal of the ether and neutralization with $NaHCO_3$, was dissolved in 10 ml of 10% NaOH and treated with 5 g *p* toluenesulphonyl chloride in 10 ml acetone. The mixture was shaken for 0.5 hr, allowed to cool for 2 hr and then poured into 50 ml water. After standing overnight, the granular solid was filtered off. This was recrystallized from 50 ml hot ethanol (yield 3.5 g, m p 75-85°), and dissolved in 150 ml methanol, on cooling, 770 mg of crude quinol di *p* toluenesulphonate m p 149-152° separated. Several recrystallizations from methanol yielded the pure ester, m p 150° and mixed m p 158° (Found C, 57.7, H, 4.15, S, 15.15 Calc for $C_{20}H_{18}O_6S_2$ C, 57.4, H, 4.3, S, 15.3 %). The methanolic filtrate from the quinol ester, when allowed to stand and evaporate, yielded 2.6 g of crude phenol *p* toluenesulphonate, m p 80°. After several recrystallizations from methanol, pure phenol *p* toluenesulphonate, m p 89° and mixed m p 90°, was obtained.

Isolation of hydroxyquinol Only very small traces of hydroxyquinol were found in urines U_1 and U_2 . It was found, however, in greater amounts in the urines of the third and fourth days (U_3 and U_4), these urines were partially hydrolyzed as described for U_1 and U_2 , and were extracted continuously with ether, U_3 for 37 hr and U_4 for 33 hr. Colour reactions showed that phenol, catechol, quinol and hydroxyquinol were present in the extracts. It was noted that hydroxyquinol tended to be extracted in the early fractions with catechol and phenol, whereas later fractions contained mainly quinol and some phenol.

The combined ether extracts of U_3 and U_4 were treated with basic lead acetate as already described for U_1 and U_2 . The lead precipitate contained the catechol and hydroxyquinol whereas the filtrate contained the phenol and quinol. The phenol and quinol fraction was on this occasion benzoylated and 200 mg of quinol dibenzoate (m p and mixed m p 196-197°) and 30 mg phenyl benzoate (m p 65-66° and mixed m p 66° Found C, 78.3, H, 5.2 Calc for $C_{13}H_{10}O_3$ C, 78.4; H, 5.1 %) were obtained.

The catechol fraction, after removal of lead, etc., was extracted with ether for 20 hr. The ether was evaporated to a reddish oil. This was neutralized with $NaHCO_3$, dissolved in 150 ml sat Na_2CO_3 solution and treated with 3 g *p* toluenesulphonyl chloride in 10 ml acetone. The mixture was shaken for 0.5 hr, kept alkaline and allowed to stand overnight. The mixture was then poured into an equal volume of water and, after standing 4 hr, the solid material (520 mg) was dissolved in 150 ml hot methanol, treated with charcoal and filtered. On cooling, 190 mg of crude catechol di *p* toluenesulphonate, m p 145°, separated. This was filtered off and the filtrate set aside to crystallize further, yielding a second crop of 220 mg of crystals, m p 95-100°, which did not depress the m p of authentic hydroxyquinol tri *p* toluenesulphonate and which gave an intense green colour on heating with conc H_2SO_4 . It was recrystallized from methanol and identified as hydroxyquinol tri *p* toluenesulphonate, m p and mixed m p 101° (Found C, 54.8, H, 4.15, S, 16.4 Calc for $C_{22}H_{24}O_6S_3$ C, 55.1, H, 4.1, S, 16.3 %). The filtrate from the hydroxyquinol ester on evaporating and cooling yielded a further 70 mg of catechol di *p* toluenesulphonate, m p and mixed m p 160° after recrystallization from methanol (Found C, 57.2, H, 4.4, S, 15.5 Calc for $C_{20}H_{18}O_6S_2$ C, 57.4, H, 4.3, S, 15.3 %).

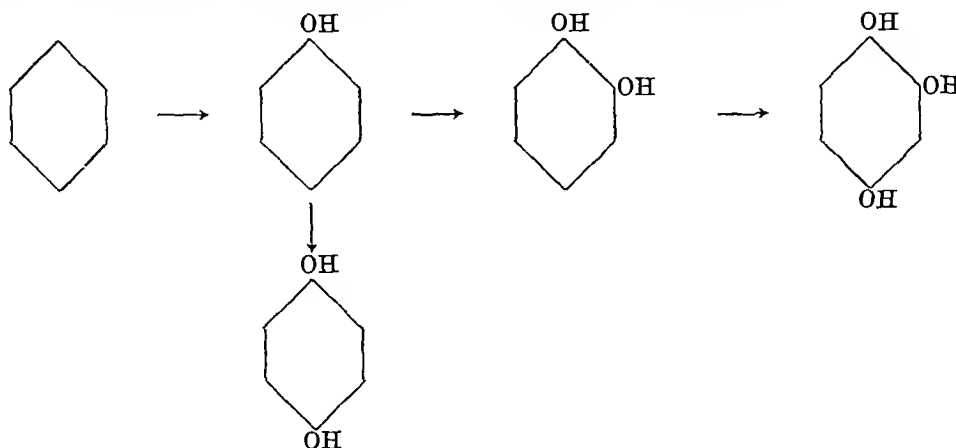
* More than half of the material was lost accidentally and the actual yield was probably at least 1.2 g

DISCUSSION

In the preceding paper (Porteous & Williams, 1949) we showed that about 9.5% of orally administered benzene is excreted by rabbits as ethereal sulphates. We have now shown that phenol, catechol, quinol and hydroxyquinol occur in the sulphate fraction of benzene urine. The yields of these phenols isolated as crystalline derivatives are given in Table 5.

The total phenols isolated amount to 3.5% of the dose of benzene. Losses in such isolation experiments are inevitably high, and we feel that the whole of the ethereal sulphate fraction, which represents 9.5% of the dose of benzene, is made up of these four phenols excreted probably as monosulphates. Other

catechol are excreted in roughly equal amounts, twice as much of each being excreted on the first 2 days as on the third and fourth days. However, the outputs of catechol, quinol and hydroxyquinol on the third and fourth days are each greater than that of phenol and most of the hydroxyquinol appears on these days. It is evident, therefore, that the first metabolite of benzene is phenol which may then be oxidized to catechol and quinol. One or both of these dihydric phenols may then give rise to hydroxyquinol. Garton & Williams (1948) have shown that hydroxyquinol is a metabolite of catechol but not of quinol (Garton & Williams, unpublished). On our present evidence the sequence in the oxidation of benzene, without reference to conjugates, may be as follows:



work in this laboratory suggests that catechol (Garton & Williams, 1948), quinol and hydroxyquinol (Anderton & Williams, unpublished) are excreted by rabbits as monosulphates. We also consider that the yield of each phenol is representative

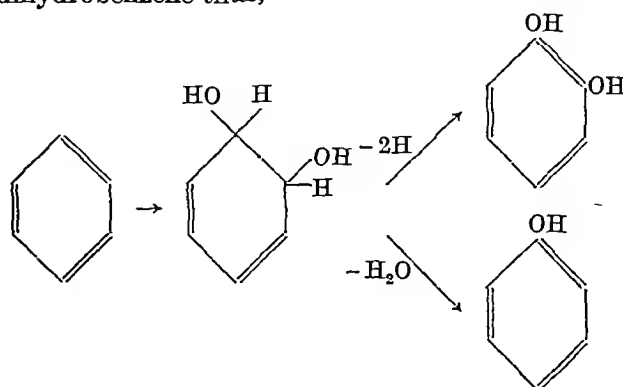
Table 5 *The recovery of phenols from the urine of rabbits receiving benzene orally*

'Phenol'	Percentage of dose isolated from		
	Urine of 1st and 2nd days	Urine of 3rd and 4th days	Total
Phenol	2.5	0.025	2.52
Quinol	0.28	0.15	0.43
Catechol	0.3	0.11	0.41
Hydroxyquinol	Traces	0.10	0.10

of the total amount of each in the ethereal sulphate fraction. Table 5 also gives some indication of the order in which these phenols are produced from benzene. On the first 2 days phenol predominates, whereas on the third and fourth days, the excretion of phenol is only about 1% of that of the first 2 days. The excretion of phenol after feeding a single dose of benzene only lasts 1 day (see Fig. 4 of the preceding paper, Porteous & Williams, 1949). Quinol and

There is, however, some uncertainty in this scheme, for we have shown (Porteous & Williams, 1949) that quinol is the main oxidation product of phenol in the rabbit, although Baumann & Preusse (1879) claim that catechol is formed in small amounts from phenol. We feel, however, that further work is necessary on the metabolism of phenol, for Baumann & Preusse (1879) have shown that potassium phenylsulphate is slightly oxidized to quinol derivatives, and it may be argued on theoretical grounds that quinol derivatives are oxidation products of phenol conjugates whereas catechol is an oxidation product of free phenol.

Phenol and catechol could arise from a dihydroxy-dihydrobenzene thus,



but as yet we have found no evidence for this scheme, which is being further considered in another publication

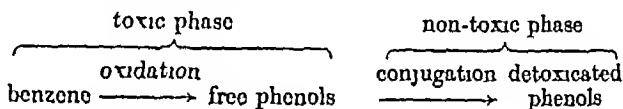
The present work has some bearing on the industrial toxicology of benzene. It is clear that the longer benzene remains in the body the greater will be the extent of its oxidation to the toxic polyhydric phenols, and continuous feeding of benzene will give rise to a continuous and increased production of catechol and quinol. The most profound effects of benzene poisoning in man are those related to the blood picture (but see Bowers, 1947). The production of erythrocytes, leucocytes and platelets in the bone marrow is affected (Bodansky & Bodansky, 1937). Thus Camp & Baumgartner (1915), from a study of the effect of benzene on abscesses in the rabbit, concluded that benzene did not reduce mature leucocytes, but only their production in the bone marrow. It is, therefore, pertinent to inquire whether the metabolites of benzene are responsible for these effects on blood cell production and the bone marrow. Oettel (1936) has shown that repeated administration of sublethal doses of quinol to cats produces an anaemia and a depigmentation of the hair. The depigmentation was considered to be due to an interference by the redox system, quinol \rightleftharpoons quinone, with enzymes of the pigment forming system. Again, Dietering (1938) has shown that repeated small doses of catechol also produce anaemia, with a reduced haemoglobin concentration, leucocyte and erythrocyte count but no depigmentation of the hair as in the case of quinol. These observations, together with our work, suggest that the effects of poisoning due to prolonged exposure to benzene may be caused by the continuous production of catechol and quinol. What part hydroxyquinol plays cannot be stated since little is known of its effects on the organism (Harold, Nierenstein & Roaf, 1910-11).

In support of the view that the toxic effects of benzene may be due to its metabolites is the recent work of Dustin on mitotic poisons. Quinol and catechol (Dustin, 1947, Parmentier & Dustin, 1948, Zylberszac, 1939) are mitotic poisons (of the trypanflavine type) which attack cells about to divide, causing nuclear destruction by pycnosis, this phenomenon is called by Dustin 'mitotic poisoning before prophase' to distinguish it from the colchicine type of mitotic poisons which arrest mitosis at the metaphase. The effect of quinol and probably of catechol is, therefore, most significant in regions of active cell proliferation such as, in the case of benzene poisoning, the bone marrow. The effects of quinol on mitosis are similar to those produced by ionizing radiations, and hence quinol and poisons of the trypanflavine type have what Dustin calls a radiomimetic effect.

According to Fabre (1947) rat-liver tissue oxidizes benzene to phenol *in vitro* more rapidly than any

other rat tissue, the optimum conditions being pH 7 and 38°. Under the same conditions toluene is not oxidized to phenolic substances. From an industrial toxicological point of view toluene is less injurious than benzene (von Oettingen, Neal, Donahue, Svrbely, Baernstein, Monaco, Valaer & Mitchell, 1942), and it is known to be metabolized entirely to benzoic acid and its conjugates (Knoop & Gehrke, 1925). This difference in toxicity between benzene and toluene and in their metabolic fates lends further support to the view that benzene is toxic because of its phenolic metabolites.

One further important observation which requires discussion is that the phenolic metabolites of benzene in the rabbit are excreted entirely conjugated with glucuronic and sulphuric acids and are, therefore, detoxicated. The phenols can only exert their toxic effect so long as they are free. There is, therefore, a toxic and a non-toxic phase in the metabolism of benzene as follows



It follows, therefore, that the toxic effects of benzene depend on the rate of its oxidation to phenols and on the rate at which the phenols are conjugated. If these rates are equal, or the process of conjugation is faster than oxidation, benzene will not exert toxic effects through the free phenols. The oxidation of benzene is probably an irreversible process, but the conjugation process may be reversed since glucuronidase and sulphatase occur in the body. Glucuronidase is known to hydrolyze glucuronides, but there is no reliable evidence that it can synthesize them (Levy, 1948). Furthermore, sulphatase hydrolyzes ethereal sulphates (Sumner & Somers, 1947), but there is no information on the synthetic ability of this enzyme. Recently it has been shown (Kerr & Levy, 1947, Levy, Kerr & Campbell, 1948) that in tissues regenerating after moderate damage by certain chemical poisons there is a marked increase in β -glucuronidase. Furthermore, cancer tissue has a very much higher glucuronidase content than the uninvolved adjacent tissue (Fishmann & Anlyan, 1947). It is possible, therefore, that under certain conditions the conjugated phenols formed from benzene may be hydrolyzed and release toxic phenols once again into the body. Conditions which would favour the occurrence of free phenols during benzene metabolism would be pathological disturbances of the liver, through either chemical poisons or an infection which would affect that organ's conjugating power or increase its β -glucuronidase content.

Damage to and impairment of the conjugating power of the liver could be brought about in a number of ways. Unchanged benzene may cause

slight damage to the liver by its solvent action on lipids. Again, a low-protein diet may affect the liver in two ways. Dogs on a low-protein diet are much more susceptible to benzene poisoning than those on an adequate protein intake (Li, Freeman, Hough & Gunn, 1945-6). Protein is apparently the main source of sulphur for detoxication of phenols as ethereal sulphates, and lack of protein thus means lack of sulphate for conjugation. In this connexion it is interesting to note that the effects of benzene poisoning in human beings treated with sulphates by injection were much milder than those in untreated cases (Ramazzotti, 1943). Furthermore, adequate protein is necessary to maintain the liver in a normal functional state. The liver may also be impaired by infections, thus upsetting its conjugating power, or increasing its β -glucuronidase content by moderate damage. In this connexion it is to be noted that in many persons there is a predisposition to benzene poisoning, and this predisposition can apparently be correlated with infections acquired prior to or during employment in a benzene-containing atmosphere (see von Oettingen, 1940 for summary, Greenburg, 1926).

SUMMARY

1 Colour reactions for detecting various phenols in mixtures and some new *p*-toluenesulphonic esters of phenols are described.

2 Phenol, catechol, quinol and hydroxyquinol have been isolated from the ethereal sulphate fraction of the urine of rabbits receiving benzene orally on two consecutive days. The phenols isolated amounted to 3.5% of the dose of benzene.

3 Phenol is excreted first and then catechol and quinol, hydroxyquinol is only found in appreciable amounts on the third and fourth days after the initial dose of benzene.

4 The results suggest the following sequence in the oxidation of benzene

benzene \rightarrow phenol \rightarrow catechol (and quinol) \rightarrow hydroxyquinol

5 The phenols excreted are almost entirely conjugated. Only traces of phenol, and none of the polyhydric phenols, are excreted in the free state.

6 The significance of the results is discussed in relation to the industrial toxicology of benzene.

NOTE ADDED 5 JULY 1948

We have recently found that catechol, quinol and hydroxyquinol are metabolites of phenol in the rabbit. Quinol is the main oxidation product, but catechol is formed in small amounts.

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The Characterization of the Esterases of Human Plasma

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The enzymes which catalyze the hydrolysis of esters of relatively low molecular weight have been divided into three main types: aliphatic esterases ('*lipases*'), which hydrolyze aliphatic esters such as methyl butyrate and tributyrin, specific or 'true' cholinesterases, which hydrolyze acetylcholine but not certain aliphatic esters, and non-specific or 'pseudo'-cholinesterases, which hydrolyze both acetylcholine and aliphatic esters. They are distinct from the lipases, which, however, may also hydrolyze the simpler triglycerides, and from other, often ill-defined esterases, such as the sterol esterases and lecithinases.

The characterization of these enzymes is made difficult both by their overlapping specificity and by the fact that two or more types of esterase may occur together in biological material. Considerable progress has, however, lately been made by the use of synthetic substrates, the hydrolysis of which appears to be specific for certain classes of esterase (Mendel, Mundell & Rudney, 1943), and by means of specific inhibitors, several very potent examples of which have been discovered in recent chemical warfare research. Thus the fluorophosphonates such as diisopropyl fluorophosphonate (DFP) inhibit pseudo-cholinesterases very powerfully and true cholinesterase to a considerably less degree (Mazur & Bodansky, 1946; Mendel & Hawkins, 1947). Thompson (1947) and Adams & Thompson (1948) have shown that another chemical warfare agent, di-(2-chloroethyl)methylamine (DDM), powerfully inhibits cholinesterases, the 'specific' cholinesterase of brain and erythrocytes being this time the more sensitive.

The differential nature of the inhibition by DFP and DDM is well brought out by measuring the ratio of the concentrations of the two inhibitors which are required to give 50% inhibition of the system under identical conditions. The values obtained for this ratio (the DDM/DFP I_{50} ratio) for the 'specific' cholinesterases of human brain and erythrocytes were of the same order of magnitude (450 and 600 respectively), the value for the cholinesterase of human plasma which, as pointed out below, is probably a non-specific cholinesterase, was widely different, being about 2.5×10^5 .

There would thus seem to be here a useful additional method for the characterization of esterases which might be used, without the necessity for enzyme purification, to decide whether a particular

esterase activity shown by different tissues is due to essentially the same enzyme or to widely different enzymes, it could also be applied to the related problem of deciding whether a number of different esterase activities shown by the same tissue is due to a single enzyme or not.

The present investigation has been made with the object of testing this second application of the method and of ascertaining what variations in the I_{50} ratio can be regarded as significant. We have chosen as our enzyme source human plasma, which has been considered by previous investigators to contain a single non-specific esterase which is uncomplicated by the presence of a second enzyme and hydrolyzes acetyl- and benzoyl-choline, glyceryl tributyrinate and triacetate, and methyl butyrate, but not acetyl- β -methylcholine.

Thus Vahlquist (1935) found that the acetylcholine and tributyrin esterase activities of human plasma were not separated by cataphoresis, that the inhibitory effects on them of atoxyl, eserine and quinine were of the same order, and that there was a significant correlation between them in different subjects, both normal and diseased, and in the same subject at different times. He also noted that methyl butyrate hydrolysis was similarly inhibited by atoxyl and eserine but was stimulated by quinine, the inhibition data were not, however, very extensive or satisfactory.

Easson & Stedman (1937), although critical of Vahlquist, agreed that acetylcholine, tributyrin and methyl butyrate esterase activities are approximately equally sensitive to eserine. They also performed summation experiments which, they state, were consistent with the conclusion that only one esterase is present. Richter & Croft (1942) confirmed and extended Vahlquist's results without, however, using any new techniques. They showed a significant correlation between the rates of hydrolysis of acetylcholine and tributyrin, and of acetylcholine and methyl butyrate, by human serum, which was unchanged by partial denaturation and absorption. They found that the hydrolysis of all three substrates was completely inhibited by $10^{-5}M$ eserine. They also confirmed the puzzling stimulating effect of quinine on methyl butyrate hydrolysis.

Mendel *et al.* (1943) found that human plasma hydrolyzes benzoylcholine. It also hydrolyzes acetyl- β -methylcholine, but only very slowly (1–2% of the rate with acetylcholine). In this it resembles the purified cholinesterase of horse serum, but differs from the enzyme of the erythrocytes and brains of a wide range of species. It is therefore classified by these workers as a pseudo-cholinesterase.

Early references to a serum lipase (e.g. Hanriot, 1898) do not, apparently, refer to a genuine lipase activity on true fats, but to the esterase activity of serum. Thus Vahlquist states that plasma is 'practically without effect on ordinary

fats' and Richter & Croft (1942) conclude that there is 'no evidence for a serum lipase in normal human serum'

The present work falls into two parts. In the first, we have used a statistical method for determining the I_{50} ratio for representative choline and non-choline esters which enables us to obtain an estimate of the probable range of the ratio for each substrate, and we have found that the values are closely similar. This and the further evidence provided by summation experiments have led us to conclude that acetyl and benzoyl-choline, tributyrin and triacetin are all hydrolyzed in plasma by one enzyme, a non-specific or pseudo-cholinesterase. In the course of this work, however, we obtained evidence for the presence in plasma of a small amount of a second enzyme, insensitive to DFP and DDM, capable of splitting aliphatic esters such as tributyrin and triacetin and also responsible for a small lipase-like activity of plasma towards such substrates as triolein. In the second part we describe a method of obtaining a preparation of the DFP-sensitive enzyme free from this second enzyme.

METHODS

Source of plasma The plasma used in most of the experiments was obtained by centrifuging fresh citrated blood provided by the E M S Blood Transfusion Service, in the remainder, the blood was drawn from laboratory volunteers, and sodium citrate (0.1%) added. Transfusion plasma was found to be an excellent enzyme source, different samples having a high and fairly constant activity. Plasma was stored at 0°, and showed little loss of activity in 3-4 weeks.

Substrates The following esters were used. Choline esters: acetylcholine chloride (ACh) (British Drug Houses Ltd), benzoylcholine bromide (BCh) (a preparation kindly furnished by Dr A H Ford-Moore, Experimental Station, Porton), aliphatic esters: glyceryl tributyrate (TB), glyceryl triacetate (TA), methyl butyrate (MeB) (all from British Drug Houses Ltd), glyceryl trioleate (TO) (as olive oil).

The choline esters were used in the form of a freshly prepared solution in 0.024M NaHCO₃, the aliphatic esters

were pipetted directly into the side bulbs of the reaction flasks and covered with bicarbonate solution. Owing to their sparing solubility they were present in the reaction mixture wholly or partly as an emulsion, the concentrations given in later sections are those that would have been attained had all the added ester dissolved. Careful purification of TB and TA before use was found to be essential, especially in the summation experiments. This was accomplished by washing with NaHCO₃ solution and water saturated with NaCl, drying over anhydrous Na₂SO₄ and distilling *in vacuo*, the middle fraction being used.

Preliminary experiments showed that under our conditions a maximum or nearly maximum rate of hydrolysis, which remained constant during the experimental period, was obtained with concentrations of ACh and BCh of 0.006M or higher, and with 0.1M TB, TA and MeB, and these concentrations were used throughout the work.

Inhibitors Di-(2-chloroethyl)methylamine hydrochloride (DDM), diisopropyl fluorophosphonate (DFP) (both from the Experimental Station, Porton). These were used as freshly prepared solutions in bicarbonate.

Estimation of esterase activity Esterase activity was determined manometrically by Ammon's (1933) adaptation of the Warburg technique, in which hydrolysis of the ester is followed by measurement of the CO₂ liberated from a CO₂/NaHCO₃ buffer at pH 7.4. The procedure was essentially that described in our previous papers (Thompson & Whittaker, 1944; Adams & Thompson, 1948), the substrate being added to the side bulbs of the Warburg flasks, and plasma, inhibitor and sufficient 0.2% NaHCO₃ to give a final volume of 3 ml to the main compartment. The flasks were then gassed with 95% N₂ + 5% CO₂, equilibrated at 38° and the substrate tipped in. Esterase activity is recorded as μ l CO₂ evolved/30 min./ml plasma.

RESULTS

Experiments with unpurified plasma

Enzymic activity of plasma Table 1 shows some collected data of the rate of hydrolysis of ACh, BCh, TB, TA and MeB in the presence of plasma. Each line of the table relates to the same plasma. The figures given are the average of different experiments, even when, as with ACh and BCh, the concentration of substrate was not always the same,

Table 1 *The relative rates of hydrolysis of ACh, BCh, TB, TA and MeB in presence of plasma esterase*
(Average values for different samples of plasma are given, the figures in parenthesis being the number of experiments averaged. Concentrations: ACh, BCh, 0.006-0.03M, TB, TA, MeB, 0.1M)

Rate of hydrolysis (μ l CO ₂ /ml. plasma/30 min.)				
ACh	BCh	TB	TA	MeB
1340 (1)	638 (2)	725 (3)	—	—
1227 (2)	555 (1)	702 (3)	—	—
1374 (6)	—	636 (6)	223 (3)	—
—	—	705 (3)*	284 (1)*	—
—	—	615 (2)	164 (2)	66 (3)
—	—	—	159 (2)	72 (2)
—	—	780 (3)	—	—
—	—	686 (8)	—	—
Av 1314	597	693	208	69

* Serum

since this was invariably such as to give a steady maximum rate of hydrolysis. The concentration of plasma varied, it was greater with the less rapidly hydrolyzed substrates in order to give conveniently measurable CO_2 evolutions.

It will be seen that ACh is the most rapidly hydrolyzed ester, that BCh and TB are split at about half, and TA and MeB at about one-seventh and one-twentieth, the rate with ACh. It should be noted, however, that the ratios of the average activities of different plasmas are not strictly comparable as there were varying losses in activity during storage.

Plasma appears to cause a small catalytic hydrolysis of triolein (Table 2), which is lost on heating, and is proportional to the amount of plasma present.

Table 2 Rate of hydrolysis of triolein by plasma

(Figures in brackets are number of experiments averaged, columns to be read independently.)

Rate of hydrolysis ($\mu\text{l CO}_2/\text{ml plasma}/20 \text{ min}$)	
0.03 u	0.07 u
33	51 (3)
27	59
33 (2)	48 (2)
—	55
—	49*
—	42

* Measured over 25 min

The rate of the catalyzed reaction was found to decrease rapidly after the substrate had been tipped into the reaction mixture, becoming nearly zero after 30 min. Changes in plasma or substrate concentration did not, under our conditions, produce a steady rate of hydrolysis, but the rate did appear to be increased when the amount of triolein was increased from 90 to 210 mmol/3 ml, even though most of the substrate was present as an emulsion. If the enzyme responsible for TO hydrolysis can act, like other lipases, at oil-water interfaces, a simple relation between substrate concentration and rate of hydrolysis cannot be expected.

Experiments with inhibitors The hydrolysis of ACh, BCh, TA and TB is inhibited very strongly by both DFP and DDM, though in much lower concentrations by the former. We have determined the I_{50} inhibitor concentrations and the I_{50} ratio for these substrates by a method which gives an estimate of the probable ranges of their values.

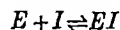
The principle is as follows. If an assumption is made as to the form of the relationship between the percentage inhibition x and the inhibitor concentration I , suitable transformations of the variables x and I into other variables x and y will give a linear relation between x and y . The line of best fit for the experimental values for x and y may now be computed. From this may be calculated the most

probable value and the range of probable values of y_{50} , the value of y corresponding to x_{50} , the value which x assumes at 50% inhibition. Suitable combinations of these y_{50} values for the two inhibitors lead to estimates of the most probable value and the range of probable values of the I_{50} ratio.

The simplest relation between x and I which appeared to fit the data is given by the expression

$$x = \frac{bI}{1 + aI} \quad (1)$$

This relation, although advanced empirically, can be derived theoretically by making certain assumptions, the chief of which is that the enzyme E and inhibitor I form a single dissociable complex EI which participates in the equilibrium



The legitimacy of applying this equilibrium to DDM and DFP is considered in the discussion.

If, in equation (1), x is put equal to $1/x$ and I to $1/y$, the expression is transformed into the linear relation

$$y = bx - a \quad (2)$$

The best estimates of b and a may then be calculated by standard statistical procedure, and from them the most probable value of y_{50} (the value of y for which $x = 1/50$), together with its standard error s , can readily be obtained.

The best estimate of the I_{50} ratio (DDM/DFP) is y_{50}/y'_{50} , where the primed symbol refers to DDM and the unprimed to DFP, the probable range of estimates of the I_{50} ratio can be obtained as follows.

The upper and lower limits of the range of y_{50} values outside which only 20% of estimates may be expected to lie is given by $y_{50} \pm ts$, where t is the appropriate value of Student's ratio corresponding to $P = 0.2$. Thus, the probability of obtaining an I_{50} ratio in which both numerator and denominator lie outside the 20% probability limits is $(0.2)^2$, and there are four such ranges of ratio, the probability of occurrence of a value within any one being $0.2^2/4$, i.e. 0.01.

These four ranges of the ratio are

$$\begin{aligned} (1) & \frac{<(y_{50} - ts)}{>(y'_{50} + t's')} & (2) & \frac{<(y_{50} - ts)}{<(y'_{50} - t's')} \\ (3) & \frac{>(y_{50} + ts)}{>(y'_{50} + t's')} & (4) & \frac{>(y_{50} + ts)}{<(y'_{50} - t's')} \end{aligned}$$

Inspection shows that (1) is the lowest range within which only 1% of estimates is likely to fall, while (4) is the highest range. The limits outside which only 2% of values of the I_{50} ratio may be expected to fall are therefore

$$\frac{y_{50} - ts}{y'_{50} + t's'} \quad \text{and} \quad \frac{y_{50} + ts}{y'_{50} - t's'}$$

ranges (2) and (3) lying between these limits.

This treatment is based on the assumption that the two y_{50} estimates are statistically independent, if they are not, the method overestimates the probable range of values of the I_{50} ratio.

Fig. 1 shows typical reciprocal plots for the inhibition of BCh by DDM and DFP, the complete data are presented in Table 3. All determinations

giving an average inhibition less than 15% have been excluded from the calculations, since it was found that inhibitions of these low values were not reproducible. Table 4 shows the most probable y_{50} values for the various substrates and inhibitors, their standard errors and the corresponding I_{50} values, and Table 5 shows the most probable values for the I_{50} (DDM/DFP) ratios, and the upper and lower limits of each ratio outside which only 2% of estimates would be expected to fall. It will be noted in Table 5 that the values for the I_{50} ratios for all substrates lie close together, any variation being well within the range of possible variation of any one substrate. On the other hand, as is shown in Table 4, the results for the I_{50} values given by either inhibitor vary considerably from substrate to substrate.

In contrast to the behaviour of the substrates so far mentioned, the triolein hydrolysis was uninhibited by both DFP and DDM, even in the highest concentrations used, $3.6 \times 10^{-6}M$ and $1.75 \times 10^{-3}M$ respectively.

Certain anomalies were noted at high DFP concentrations. Concentrations which were considerably in excess of those required to produce a practically complete inhibition of ACh hydrolysis failed to do so with the aliphatic substrates, at any rate during the first 10 min of the measurements, i.e. until after about 35 min after mixing enzyme

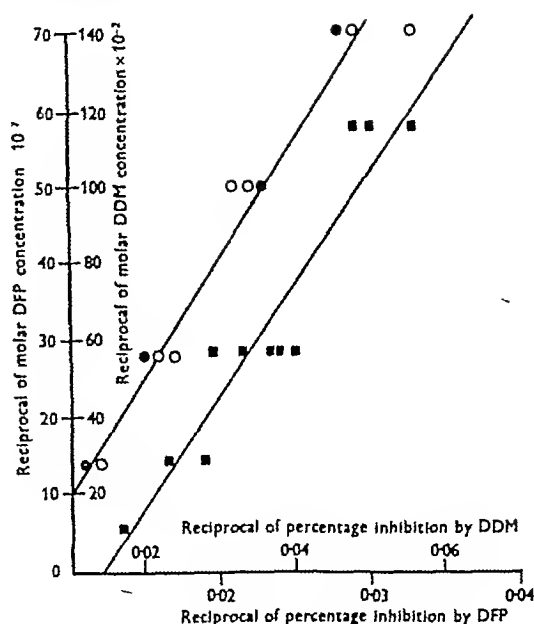


Fig 1 The inhibition by DFP and DDM of the hydrolysis of 0.006M-benzoylcholine by human plasma (0.2/3 ml). Ordinates reciprocal of inhibitor concentrations, abscissae reciprocal of percentage inhibition. The straight lines are the regressions of y on x . Circles experiments with DFP, black circles denoting two coincident points, squares experiments with DDM. (DFP = diisopropyl fluorophosphate, DDM = di-(2-chloroethyl)-methylamine.)

Table 3 The inhibition of plasma esterases by DFP and DDM

(Concentration of plasma ACh, BCh and TB experiments, 0.2 ml/3 ml, TA experiments, 0.5 ml/3 ml. Concentration of substrates ACh, BCh, 0.006M, TB, TA, 0.1M)

A Inhibition by DFP over 30 min

DFP concentration $\times 10^9$ (M)	(Percentage)			
	ACh	BCh	TB	TA
0.9	—	—	28*, 27, 27	—
1.0	—	—	—	20, 29
1.42	—	30, 35, 36, 36	—	—
1.8	34, 34, 37	—	51, 53, 54*	—
2.16	—	43, 44, 46, 47	—	—
3.0	—	—	—	45, 46
3.6	56, 56, 60	60, 61, 65, 67	71, 73*, 83	—
5.0	—	—	—	55, 59, 66
5.4	76	—	—	—
7.1	83, 89	86, 87, 90	83*, 84, 89	—
10.1	97	—	—	—
50.0	—	—	—	81

B Inhibition by DDM over 30 min

DDM concentration $\times 10^4$ (M)	(Percentage)			
	ACh	BCh	TB	TA
0.87	—	18, 20, 21	15, 17, 20	—
1.75	11, 13, 13, 16, 18, 19	25, 26, 27, 30, 34	22, 26, 28	17, 20, 22
3.5	29, 33, 33, 34	36, 44	39, 41, 44, 51	33, 34, 34
6.1	45, 47	—	—	—
7.0	48	—	56, 56, 58, 59	—
8.7	52, 54, 56	58	—	47, 50, 52
14.0	—	—	70	—
17.5	59, 63, 64	—	—	57, 58, 64

* Serum

Table 4 I_{50} values for DDM and DFP(Units y_{50} values, DFP series, $M^{-1} \times 10^{-7}$, DDM series, $M^{-1} \times 10^{-2}$, I_{50} values, DFP series, $M \times 10^3$, DDM series, $M \times 10^4$)

Substrate	No of obser vations	y_{50}	Standard error (s)	Range of y_{50} for which $P=0.2$		I_{50} values and limits outside which less than 20% of estimates would be expected to fall		
				$y_{50} \pm ts$	$y_{50} - ts$	Upper	Most probable	Lower
				DFP				
ACh	10	34.85	1.808	37.50	32.20	3.11	2.87	2.67
BCh	15	41.47	3.345	45.99	36.95	2.71	2.41	2.18
TB	12	50.50	1.706	52.84	48.16	2.08	1.98	1.89
TA	8	30.42	4.690	37.06	23.78	4.21	3.29	2.70
DDM								
ACh	10	15.31	3.264	19.66	10.96	9.12	6.53	5.09
BCh	11	17.41	1.194	19.06	15.76	6.34	5.74	5.24
TB	15	20.67	3.321	25.15	16.19	6.18	4.83	3.98
TA	12	12.48	1.803	14.95	10.01	9.99	8.01	6.69

Table 5 I_{50} (DDM : DFP) ratios for ACh, BCh, TB and TAMost probable value of I_{50} ratio ($\times 10^{-5}$) and limits outside which less than 2% of estimates would be expected to fall

Substrate	Upper limit	Most probable value	Lower limit
ACh	3.42	2.28	1.64
BCh	2.92	2.38	1.94
TB	3.26	2.44	1.91
TA	3.70	2.44	1.59

non-specific enzyme, however, the rate of reaction of the mixed substrates will in no case exceed the greater of the rates of the separate reactions

Table 6 Effect of $10^{-7}M$ DFP on plasma esterase

(a=0-10 min, b=10-30 min)

Activity (μ l CO ₂ /ml plasma)				Inhibition (%)	
(a)		(b)		(a)	(b)
Control	Inhibited	Control	Inhibited		
ACh (0.02M)					
463	11	528	3	97.5	99.5
507	0.5	973	8	100	99
450	0	900	8	100	99
465	5	845	2	99	99.5
TB (0.1M)					
113	13	352	11	88	97
210	26	415	11	88	97
200	10.5	450	14	95	97
270	22	400	13	88	97
TA (0.1M)					
68	20	148	10	71	93
71	22	157	1	69	99

and inhibitor. This is illustrated in Table 6, in which the inhibition produced by $10^{-7}M$ -DFP on TB and TA hydrolysis is compared during an initial 10 min period and during a subsequent 20 min period with the inhibition of ACh hydrolysis. It will be seen that the inhibition by DFP is by no means complete with the aliphatic substrates, though $10^{-6}M$ -DFP would probably have been sufficient to produce prompt and complete inhibition of ACh hydrolysis.

The time course of this inhibition of the aliphatic substrates closely parallels the time course of the TO esterase activity and suggests that the triolein enzyme may be responsible for the small amount of ali-esterase activity which escapes inhibition.

Summation experiments Having concluded from our determinations of the I_{50} ratios that one enzyme is mainly responsible for the hydrolysis of both aliphatic and choline esters, we sought confirmatory evidence by means of summation experiments.

A consideration of the kinetics of enzyme reactions with mixed substrates leads to the following conclusion. If two enzymes are present, each specific for one substrate, the rate of reaction of the mixed substrates will be equal to the sum of the rates of the separate reactions, provided neither substrate acts as an inhibitor of the other's enzyme. With a single

Table 7 shows the results of summation experiments carried out with ACh and TB, BCh and TB, and BCh and TA. Columns 3 and 4 give the rates of enzymic hydrolysis of ACh, BCh, TB and TA alone, column 5 that of the mixed substrates, ACh+TB, BCh+TB or BCh+TA. It will be seen that in each case the rate of hydrolysis of the mixed substrates is almost equal to the greater of the rates of hydrolysis of the single substrates, and is very much below the sum of these rates (column 6). These results confirm, therefore, the interpretation placed on the results of the inhibitor experiments, namely, that the ali-esterase and cholinesterase activities of human plasma are mainly due to a single enzyme.

Table 7 *A comparison between the rates of hydrolysis of mixed aliphatic and choline ester substrates and those of the esters alone*

(Concentration of plasma TB experiments, 0.2 ml / 3 ml, TA experiments, 0.5 ml / 3 ml)

Concentration (M)		Rate of hydrolysis (μ l CO ₂ /30 min.)				
Choline ester	Aliphatic ester	Choline ester (a)	Aliphatic ester (b)	Mixed esters (c)	(a + b)	c less a or b
ACh + TB						
0.006	0.1	232	155	246	387	14
0.015	0.1	259	137	275	396	16
0.03	0.1	268	142	288	410	20
0.03	0.1	299	155	317	454	18
0.03	0.1	314	147	319	461	5
0.03	0.1	282	136	296	418	14
0.03	0.1	288	148	300	436	12
BCh + TB						
0.006	0.1	111	129	144	240	15
0.015	0.1	130	153	175	283	22
0.03	0.1	125	140	147	265	7
BCh + TA						
0.03	0.1	312	151	337	463	25
0.03	0.1	318	139	345	457	27

It will be noted, however, that the values obtained for the rate of hydrolysis of the mixed substrates are slightly, but consistently, greater than the corresponding values for the greater of the rates of the single substrates. * The difference is recorded in the last column and indicates a small degree of positive summation. It suggests that at least 10% of the tributyrin esterase activity of the plasma and 17% of the triacetin activity is due to a second enzyme, and taken in conjunction with the low, but reproducible, activity of plasma towards triolein, and with the inhibition experiments with 10^{-7} M-DFP, implies that the DFP-insensitive triolein esterase contributes to the total TB and TA esterase activity of plasma.

Further support for this view was obtained in summation experiments with tributyrin and triolein in the presence of a concentration of DFP sufficient to inhibit the DFP-sensitive esterase. The results recorded in Table 8 show that the DFP-insensitive hydrolysis of TB and TO is not additive, the sum of the rates of hydrolysis of TB and TO alone being much greater than the rate of hydrolysis of the mixed substrates. The presence of a small variable positive summation may be due partly to the difficulty of making accurate measurements with these low rates of CO₂ evolution, and partly to the impossibility of knowing the exact concentration of DFP required to produce complete inhibition of the one enzyme while leaving the other unaffected.

* The effect is not obtained unless recently purified aliphatic esters are used. The significance of this is not clear, perhaps the aged esters contain an inhibitory substance as noted by Mendel & Rudnev (1943).

Table 8 *A comparison, in the presence of 10^{-7} M-DFP, of the hydrolysis of a mixture of TB and TO with that of each substrate alone*

(Concentration of substrates, 0.1 M)

Rate of hydrolysis (μ l CO ₂ /ml. plasma/60 min.)			
TB (a)	TO (b)	Mixed substrates	(a + b)
54	51	60	105
52	70	73	122
48	62	84	110
32	53	56	85

The conclusion is thus strengthened that there exists in human plasma a small amount of a DFP-insensitive lipase-like enzyme which contributes to the total aliphatic esterase activity of the plasma.

Experiments with purified plasma esterase

Having demonstrated in our experiments with unpurified plasma that a DFP-insensitive lipase-like enzyme contributes significantly to its total aliphatic esterase activity, we next attempted to remove it by purification procedures. The method finally employed was ammonium sulphate fractionation at pH 6.

Procedure All operations were carried out at $0 \pm 2^\circ$ to avoid the otherwise rapid inactivation of the preparations.

Sulphate and protein (as dry wt) in active fractions were determined as follows. The fraction (0.5 ml) was pipetted into 15% (w/v) trichloroacetic acid and the mixture just brought to the boil. The protein precipitate was removed by filtration through paper and washed, the sulphate in the filtrate and washings was then determined gravimetrically.

as BaSO_4 . The protein precipitate was washed off the paper into the crucible on top of the BaSO_4 , the whole being redried and reweighed. $(\text{NH}_4)_2\text{SO}_4$ concentrations are recorded as percentages of the concentration of the saturated solution at 0° (50% w/v). The progress of the purification was checked by determining activity/mg protein (dry wt). A typical preparation was as follows.

Fresh human plasma (200 ml) was brought to 60% saturation by the addition during 2 hr of an equal vol of saturated $(\text{NH}_4)_2\text{SO}_4$ solution followed during a further hour by 21 g of solid $(\text{NH}_4)_2\text{SO}_4$. After standing overnight, the inactive precipitate was removed by centrifuging. The supernatant liquid was brought to 71% saturation by the slow addition of a further quantity of solid $(\text{NH}_4)_2\text{SO}_4$ and the mixture again allowed to stand overnight. A second precipitate, containing most of the enzymic activity, was collected by centrifuging and dissolved in glass distilled water to 100 ml. After determining the concentration of $(\text{NH}_4)_2\text{SO}_4$ already present, more solid salt was gradually added to give 70% saturation. After 4 hr the precipitate was collected and redissolved in water (60 ml). This procedure was repeated twice, the fraction appearing between 65 and 69.5% saturation being collected in the first reprecipitation and that between 66.5 and 69.5% in the second. The final preparation was taken up in water (30 ml), $(\text{NH}_4)_2\text{SO}_4$ removed by dialysis and sufficient bicarbonate added to give 0.2% concentration.

Of the original activity 25–30% was present in the purified preparation, which had a cholinesterase activity/mg protein (dry weight) 16–20 times that of the original plasma, and a negligible triolein esterase activity. Precipitation of the active fraction between 66.5 and 69.5% saturation, i.e. between the globulin and albumin fractions, accords with Vahlquist's (1935) cataphoresis experiments.

Inhibition experiments. Table 9 shows the effect of 10^{-7}M -DFP on the acetylcholine and tributyrin hydrolysis by a purified plasma esterase preparation. The results obtained with an unpurified portion of

the same plasma are included for comparison. It will be seen that, whereas in the unpurified plasma (as in the more extended experiments of Table 6) the inhibitory effect of 10^{-7}M -DFP on the TB esterase activity is somewhat less than that on the ACh esterase activity during the first 10 min period, with the purified preparation, in which there is no triolein activity, the inhibition with both substrates is the same and essentially complete.

Summation experiments. The removal of the triolein esterase from plasma is also associated with the disappearance of the small degree of positive summation noted in a previous section when the rate of

Table 10 *Summation experiments with aliphatic and choline esters*

(Concentration of substrates ACh and BCh, 0.03M, TB and TA, 0.1M)

CO ₂ evolution ($\mu\text{l}/30\text{ min}$)			
(a) Choline ester	(b) Aliphatic ester	(c) Mixed esters	(c - a)
ACh + TB, unpurified plasma (0.18 ml/3 ml)			
282	136	206	+14
288	148	300	+12
ACh + TB, purified fraction from same plasma (0.15 ml/3 ml)			
276	126	276	0
272	120	271	-1
277	132	275	-2
BCh + TA, unpurified plasma (0.5 ml/3 ml)			
312	151	337	+25
318	139	345	+27
BCh + TA, purified plasma (0.5 ml/3 ml)			
317	111	313	-4
316	105	306	-10
328	109	320	-8

Table 9 *A comparison, with unpurified and purified plasma esterases, of the inhibitory effect of 10^{-7}M -DFP on TB and ACh hydrolysis*

Enzyme activity ($\mu\text{l CO}_2$)				Inhibition (%)	
0–10 min		10–30 min		0–10 min	10–30 min
Control	Inhibited	Control	Inhibited		
A Unpurified plasma ($\mu\text{l}/\text{ml}$)					
ACh (0.02M)					
450	0	900	8	100	99
465	5	845	2	99	100
TB (0.1M)					
200	11	450	14	95	97
270	22	400	13	88	97
B Purified plasma ($\mu\text{l}/\text{ml equiv}$)					
ACh (0.02M)					
365	1.5	795	6	99.5	99
505	2	860	3.5	99.5	99.5
TB (0.1M)					
185	1	325	6	100	98
240	4	400	4	98.5	99

different estimations, without hydrolysis and after hydrolysis with N HCl, N -NaOH and $10N$ -NaOH with addition of urea

To develop the colour in each solution the pH was adjusted to 5.5 with KH_2PO_4 and NaOH using bromocresol purple as external indicator. 4 and 8 ml, respectively, of these solutions were used, the former being made up to 8 ml with water, the solutions were heated to 75–80° and 0.8 ml of freshly prepared CNBr was then added in a test tube graduated at 10 ml, the solution was heated to 75–80° for another 5 min. After cooling to room temperature the volume was made up to 10 ml with water and 0.4 g *p*-methylaminophenol sulphate (metol) was added and allowed to dissolve completely. Blanks were developed simultaneously by adding to similar volumes of urine 0.15 ml N H_2SO_4 instead of the metol. Standards were made from solutions containing 2 and 4 μg /ml, respectively of nicotinic acid. After 1 hr in the dark the concentration was measured by comparing the extinction with that of the standard in a Bausch and Lomb 'Colorimeter' used as a photometer with a Wratten filter no. 39.

On an equimolecular basis, nicotinamide (*Nam*) gave 50% of the extinction given by nicotinic acid (*Nac*) before and 100% after hydrolysis with N -HCl or N -NaOH. Nicotinic acid (*Nur*) gave values of 30% before hydrolysis and no increase after hydrolysis with N HCl, but 100% after hydrolysis with N NaOH. Trigonelline (*Trig*) and nicotinamide methochloride (*NM*) gave no coloured compounds before or after hydrolysis with N acid, N NaOH or $10N$ NaOH. After hydrolysis with $10N$ -NaOH, in the presence of urea, a colour equivalent to 63% of that of nicotinic acid was obtained.

Calculation of the concentrations of the different metabolites was carried out as follows. If the value obtained before hydrolysis is called *A*, that after hydrolysis with acid *B*, that after hydrolysis with N NaOH *C*, and that after hydrolysis with $10N$ -NaOH in the presence of urea *D*, then these equations follow from the figures given above

$$A = 1 \text{ Nac} + 0.5 \text{ Nam} + 0.3 \text{ Nur},$$

$$B = 1 \text{ Nac} + 1 \text{ Nam} + 0.3 \text{ Nur},$$

$$C = 1 \text{ Nac} + 1 \text{ Nam} + 1 \text{ Nur},$$

$$D = 1 \text{ Nac} + 1 \text{ Nam} + 1 \text{ Nur} + 0.63 (\text{Trig} + \text{NM}),$$

$$\text{Nam} = 2 (B - A),$$

$$\lambda_{ur} = \frac{C - B}{0.7},$$

$$\lambda_{ac} = C - 2 (B - A) - \frac{C - B}{0.7},$$

$$\text{Trig} + \text{NM} = \frac{D - C}{0.63},$$

λM is estimated separately by a method described later, and

$$\text{Trig} = \frac{D - C}{0.63} - \lambda M \text{ (in terms of Nac)}$$

Since all values are obtained in terms of nicotinic acid, the values have to be multiplied by the mol wt of the metabolite and divided by that of nicotinic acid. The values obtained in that way in terms of μg /ml are multiplied by the urine volume to obtain the value of the 24 hr elimination. The maximum error of the method, as estimated from recovery tests of each of the metabolites added to urine, is in the region of $\pm 10\%$, when all metabolites are present,

when only nicotinic acid and nicotinamide have to be estimated, the maximum error found was less than $\pm 5\%$. The smallest concentration which can be estimated by this method is 0.8 μg /ml.

Nicotinamide methochloride was estimated by a modification of the acetone method of Huff & Perlzweig (1947), since non fluorescent KCl was unobtainable and the method of Coulson *et al* (1944) could thus not be used. The procedure differed from that described by Huff & Perlzweig in one respect: the fluorescence was compared visually with that of standards prepared from synthetic nicotinamide methochloride in a similar manner. Recoveries of 95–105% were obtained with added nicotinamide methochloride. Filter paper was chosen which neither adsorbed nicotinamide methochloride nor released fluorescent pigments, the charcoal used adsorbed interfering pigments, but not nicotinamide methochloride. Acetone was freed from fluorescent pigments by distillation over $KMnO_4$. The maximum error was about $\pm 5\%$. The range of the method was found to be 0.01–1.0 μg /ml. Urines containing higher concentrations were diluted before the estimation. All analyses were carried out in duplicate. Negative or dubious results obtained with the König reaction were checked microbiologically with the method of Barton-Wright (1944) using *Lactobacillus arabinosus*. The presence of the coenzymes which give a positive reaction with the acetone method was checked microbiologically by the method of Pittman & Fraser (1940) using *Haemophilus parainfluenzae* (National Collection of Type Cultures, no. 4101).

RESULTS

Man. In man (Table 2a) nicotinamide methochloride is the main end product of nicotinamide metabolism forming on the average 71.2%, whilst nicotinamide represents 24.2% and nicotinic acid 4.6% of the output. These values may be compared with those of Johnson *et al* (1945), who found, however, absolute values for nicotinamide methochloride (average 18.7 mg) far higher than either those found in the present work or any values published by previous investigators (Ellinger & Coulson, 1944; Ellinger, Benesch & Hardwick, 1945; Hochberg, Melnick & Oser, 1945; Ellinger & Hardwick, 1947). Ingested nicotinic acid (Table 2b) is amidated and methamphetamine is de-ethylated. In all cases nicotinamide methochloride forms the main metabolite. After subcutaneous administration the elimination is greater than after oral administration, in agreement with the findings of Ellinger & Hardwick (1947). The total recovery of the ingested compounds is much lower than in any other mammal examined. This might be due to the fact that part of the methylated compound is immediately oxidized to the corresponding pyridone (Rosen, Perlzweig & Handler, 1948) and not determined by the methods of assay used. It might also be due to a relatively low saturation state of the subject examined, indicated by the considerable rise in output of the three metabolites in the course of administration of nicotinamide derivatives. Similar observations have

Table 2a *Daily elimination of nicotinamide derivatives by man*

Subject				Metabolites eliminated (mg /24 hr)			Remarks
No	Sex	Age (yr)	Wt (kg)	Nicotinamide	Nicotinic acid	Nicotinamide methochloride*	
1†	♂	27	80	2 000	0 810	8 630	No smoking, no coffee
R1‡	♂	27	80	1 342	0 360	6 081	
R1§	♂	27	80	3 622	1 233	11 420	
2	♀	61	50	3 320	0 680	6 160	No smoking, coffee
3	♀	19	70	1 400	0 480	9 360	
4	♀	19	65	2 180	0 370	9 440	No smoking, no coffee
5	♀	47	67	1 980	0 215	12 040	
6	♀	22	70	1 590	0 264	12 480	
7	♀	25	58	1 175	0 190	10 840	
8	♂	53	64	2 540	0 145	6 050	
9	♂	60	68	3 000	0 440	5 040	Balanced diabetic, no smoking, no coffee
Average				2 132	0 399	8 894	

Neither trigonelline nor nicotinic acid were eliminated

* Absolute, not in terms of nicotinic acid

† Average of daily output estimated on 22 control days spread over the whole period

‡ Average of 3 days before the first injection

§ Average of 3 days, 14 days after last injection

Table 2b *Response to extraductary nicotinamide derivatives by man (subject no 1)*

Compound administered	Dose (mg)	Route	Metabolites eliminated (% of ingested)*			
			Nicotinamide	Nicotinic acid	Nicotinamide methochloride	Total
Nicotinamide	100	Oral	0	0	18.9 [3]	18.9
		Subcutaneous	1.8	0	24.9 [3]	26.7
Nicotinic acid	100	Oral	2.3	0.8	16.5 [2]	19.6
		Subcutaneous	2.1	0.9	21.1 [2]	24.1
Nicotindimethylamide	100	Oral	6.2	0	5.8 [2]	12.0
		Subcutaneous	6.3	0	15.5 [2]	21.8

[] indicates time in days over which elimination occurred

* Note Dose of administered compound is in terms of nicotinic acid. The response (percentage of ingested), also in terms of nicotinic acid, is calculated as

$$\frac{(\text{output until predosing level is reached}) - (\text{average predosing output} \times \text{days of increased output})}{\text{dose administered}} \times 100$$

Neither trigonelline nor nicotinic acid was eliminated

been made by Ellinger & Hardwick (1947). The smaller elimination of metabolites after oral than after parenteral administration indicates destruction by the intestinal flora which has been observed by Koser & Baird (1944), Benesch (1945), Ellinger & Emmanuelowa (1946), Ellinger (1947) and Ellinger, Abdel Kader & Emmanuelowa (1947).

Dog In this species (Table 3) nicotinamide and nicotinamide methochloride are the only urinary metabolites, the relative proportion of the two compounds eliminated differed considerably in the two animals examined, but was fairly constant from day to day in the same animal. The total elimination was high compared with that of other species. After administration of nicotinamide and nikethamide (Table 4), the only metabolites found were nicotinamide and nicotinamide methochloride, while after nicotinic acid injection some unchanged nicotinic

acid appeared in the urine. This, and particularly the absence of nicotinic acid, contradicts the findings of Ackermann (1913), Komori & Sendju (1926) and Sarett (1942). The last named found a recovery of 75% in the form of 'trigonelline' and 25% in that of nicotinic acid. When Ackermann's (1913) experiment was repeated, neither dog eliminated either trigonelline or nicotinic acid. Dogs gave a much higher response to injected compounds than man. The relatively greater elimination of the methylated product with smaller than with larger doses suggests that the methylating mechanism is the limiting factor. The relative proportion of the methylated to the unmethylated metabolites is considerably greater after administration of nicotinamide and nikethamide than after that of nicotinic acid. The relative total response is greater after small than after big doses.

Table 3 *Average daily urinary elimination of nicotinamide derivatives by various mammalian species*

Species	Metabolites eliminated (Averages, limiting values in parentheses) (mg/day)		
	Nicotinamide	Nicotinic acid	Nicotinamide methochloride
Dog	4 590 (3 480-5 720)	0	5 370 (1 000-10 250)
Cat	0	0	0 290 (0 070-0 400)
Rat (albino)			
Adult	0 080 (0 054-0 130)	0	0 100 (0 030-0 280)
Young	0 035	0	0 292
Rat (hooded)			
Adult	0 140 (0 100-0 170)	0	0 960 (0 450-1 700)
Young	0 0214	0	0 150
Rabbit	0	0 880	0*
(On cabbage)		(0 500-1 350)	
(On oats)	0	1 190 (1 010-1 540)	0*
Guinea pig	0	0	0*

Neither trigonelline nor nicotinuric acid were eliminated

* Indicates a small positive reaction with the acetone, but none with the König (1904) method

Table 4 *Average response to extradietary nicotinamide derivatives by dogs*

Compound administered	Dose* (mg)	Metabolites eliminated (% of ingested)*			Total
		Nicotinamide	Nicotinic acid	Nicotinamide methochloride	
Nicotinamide	200	31.7 [2]	0	50.1 [2]	81.8
	500	43.0 [2]	0	31.6 [3]	74.6
Nicotinic acid	200	48.8	13.1	31.6 [2]	93.5
	500	49.0 [2]	16.7	21.5 [2]	87.2
	9000†	10.0	49.4	5.6	65.0
Nicotindimethylamide	100	43.5	0	52.1 [2]	95.5
Nicotinuric acid	67.7	31.4	41.8	18.1 [2]	91.2

[] indicates time in days over which elimination occurred, if more than one

Neither trigonelline nor nicotinuric acid were eliminated

* See note to Table 2b

† Orally within 5 days

Table 5 *Average response to extradietary nicotinamide derivatives by cats*

Compound administered	Dose* (mg)	Metabolites eliminated (% of ingested)*			Total
		Nicotinamide	Nicotinic acid	Nicotinamide methochloride	
Nicotinamide	40	34.5	0	28.3 [3]	63.3
	80	37.6 [2]	0	20.4 [2]	58.0
	200	47.0 [2]	0	34.1 [4]	81.1
Nicotinic acid	40	12.8	10.5	24.1 [2]	47.4
	100	24.1 [2]	29.0	18.7 [3]	71.8
Nicotindimethylamide	40	19.0	0	16.1 [2]	35.0

[] indicates time in days over which elimination occurred, if more than one

Neither trigonelline nor nicotinuric acid were eliminated

* See note to Table 2b

Cats normally eliminate small amounts of nicotinamide methochloride only (Table 3) Nicotinamide and nicotinamide methochloride were eliminated after administration of nicotinamide and nikethamide and in addition some nicotinic acid when this compound was given (Table 5) As in dogs, the relative proportion of methylated to unmethylated metabolites decreases with rising doses The total response was lower than in dogs

similar to those observed by Huff & Perlzweig (1942), though the experiments were not directly comparable Nicotinuric acid was found to be split as observed by Huff & Perlzweig (1942), but not so completely as by dogs Nicotinamide methochloride was eliminated unchanged but only to the extent of 63 %, confirming the findings of Ellinger (1947), Ellinger & Coulson (1944) and Perlzweig & Huff (1945) in man The effect of methionine administered,

Table 6 *Response to extradietary nicotinamide derivatives by two strains of rats*

Compound administered	Dose* (mg)	Metabolites eliminated (% of ingested)*				
		Nicotinamide	Nicotinic acid	Nicotinamide methochloride	Nicotinuric acid	Total
Albino						
Nicotinamide	20	23.7	0	9.3	0	33.0
	20 (+30 DL-methionine)	19.2	0	12.5	0	31.7
	50	31.6	0	15.0 [2]	0	46.6
Nicotinic acid	20	23.8	26.9	1.8	0	52.5
	20 (+30 DL-methionine)	20.4	49.5	1.0 [2]	0	70.9
	50	31.9 [2]	35.4	1.0 [2]	0	68.3
Nicotindimethylamide	20	36.7	0	9.8 [2]	0	46.5
	20 (+30 DL-methionine)	48.0	0	4.9 [2]	0	52.9
	50	38.8 [2]	0	3.4 [2]	0	42.2
Nicotinamide methochloride	17.5	0	0	71.0	0	71.0
Nicotinuric acid	33.9	13.0	2.1	3.2 [2]	72.8	91.0
Hooded						
Nicotinamide	20	15.0	0	29.7	0	44.7
	20 (+30 DL-methionine)	16.5	0	59.7	0	76.2
	50	19.2	0	61.6 [2]	0	80.8
Nicotinic acid	20	24.1	27.7	16.0	0	67.8
	20 (+30 DL-methionine)	26.0	37.8	8.9 [2]	0	72.7
	50	25.4	19.4	5.7	0	50.5
Nicotindimethylamide	20	36.0	0	33.4 [2]	0	69.4
	50	34.4 [2]	0	13.7 [2]	0	48.1
Nicotinamide methochloride	17.5	0	0	63.2	0	63.2
Nicotinuric acid	33.9	11.4	2.0	3.1 [2]	62.2	79.6

[] indicates time in days over which elimination occurred, if more than one

No trigonelline was eliminated

* See note to Table 2b

Rat The two strains of rat examined differed mainly in the efficiency of the methylating mechanism. Rats, like dogs, eliminated only nicotinamide and nicotinamide methochloride (Table 3) The relative proportion of the latter to the former was 1.25 in the albino and 6.85 in the hooded strain. Relative to weight the output is high compared with that of cats, for the albino it was similar to that found by Huff & Perlzweig (1942) and for the hooded strain it was much higher. Both strains eliminated injected nicotinamide and nikethamide as nicotinamide and nicotinamide methochloride, when nicotinic acid was given it was eliminated partly unchanged (Table 6) The total response was greater in the hooded than in the albino strain. The values found for response to nicotinamide and nicotinic acid were

together with the nicotinamide derivatives on the formation of nicotinamide methochloride, was irregular. The elimination of nicotinic acid after administration of nicotinic acid was always increased by simultaneous administration of methionine. When large doses (100 mg) of nicotinamide were administered the nicotinamide methochloride output was increased by rising doses of methionine, this indicates the exhaustion of methyl donors by methylation as shown for man by Ellinger & Hardwick (1947). In all cases the elimination of nicotinamide methochloride was far greater in the hooded than in the albino strains. On no occasion was nicotinamide deaminated or nicotinic acid formed from nikethamide. DL-Methionine proved to be toxic to rats. LD₅₀ found was about 700 mg/kg. The hooded rats

died earlier and were more strongly affected by sublethal doses than the albino. Macroscopic post-mortem examination showed the kidneys congested with blood, confirming observations by Simmonds, Cohn & du Vigneaud (1947).

Young rats (Table 3), like adults, eliminated nicotinamide and nicotinamide methochloride but no nicotinic acid. This conversion of nicotinic acid into nicotinamide makes the observation of Handler & Dann (1942) that the growth of young rats is inhibited by nicotinamide but not by nicotinic acid even less explicable.

tinamide methochloride. It is concluded, therefore, that a substance is present in the urine giving the acetone but not the König reaction. A test for co-enzymes which fulfils this condition gave negative results. The possibility has, therefore, to be considered that there is present an unknown factor which might be a metabolite of a nicotinamide derivative. It will be extremely difficult to isolate or identify it since it is present in minute quantities and no way has been found to increase its elimination markedly. It is possible that the ' P_2 ' which, according to Handler (1944), is eliminated by young

Table 7 Average response to extradietary nicotinamide derivatives by rabbits

Compound administered	Dose* (mg)	Metabolites eliminated (% of ingested)*			
		Nicotinamide	Nicotinic acid	Trigonelline	Total
		Diet of cabbage			
Nicotinamide	100	2.9	3.3 [2]	5.1 [2]	11.3
	200	6.9	14.5 [3]	9.3 [3]	30.6
	400	7.5	18.6 [3]	9.4 [3]	35.5
Nicotinic acid	100	0	35.5 [2]	48.0 [2]	83.5
	200	0	63.4 [2]	33.9 [2]	97.3
Nicotindimethylamide	50	1.6	5.7	3.3	10.6†
	100	9.2	15.1	6.4	30.7†
	200	4.9	10.0 [2]	7.0 [2]	21.8†
	2 hourly doses of 100				
	600	7.5	21.7 [2]	2.7 [2]	30.9†
6 hourly doses of 100					
Diet of oats					
Nicotinamide	100	2.2	3.5 [2]	3.3	9.5‡
	200	7.5 [2]	13.0 [3]	6.5 [3]	27.0‡
Nicotinic acid	100	0	32.9 [2]	54.8 [2]	87.7
	200	0	52.4 [2]	29.8 [2]	82.2

[] indicates time in days over which elimination occurred, if more than one.

Neither nicotinamide methochloride nor nicotinuric acid were eliminated.

* See note to Table 2b.

† Indicates that after injection of nicotindimethylamide an unknown substance of high fluorescent efficiency was eliminated interfering with the application of the acetone method.

‡ Indicates that after injection of nicotinamide into rabbits fed on oats and possibly after that of nicotindimethylamide a substance was eliminated to a slightly increased extent giving a positive reaction with the acetone but not with the König (1904) method.

Rabbits (Table 3) independently of the diet, cabbage or oats, normally only eliminated nicotinic acid. In addition a substance was regularly eliminated, and to a slightly increased extent after injection of nicotinamide by rabbits fed on oats, which gave a positive reaction by the acetone method for nicotinamide methochloride. The amount was very small, just within the limits of the method, but the substance appeared to be always present. The König (1904) assay failed to show the presence of trigonelline or nicotinamide methochloride in the same urines. This might be due to insufficient sensitivity of the König reaction, however, this is improbable. The concentrations found with the acetone method were large enough to give positive results with the König method if the substance were nico-

tinamide methochloride. It is concluded, therefore, that a substance is present in the urine giving the acetone but not the König reaction. A test for co-enzymes which fulfils this condition gave negative results. The possibility has, therefore, to be considered that there is present an unknown factor which might be a metabolite of a nicotinamide derivative. It will be extremely difficult to isolate or identify it since it is present in minute quantities and no way has been found to increase its elimination markedly. It is possible that the ' P_2 ' which, according to Handler (1944), is eliminated by young

rabbits and guinea pigs, but not increased after nicotinamide administration, and the nicotinamide methochloride found by Johnson *et al.* (1947) in the urine of young calves, which also is not increased by administration of nicotinamide, is in fact this unknown substance.

Extradietary nicotinamide and nikethamide were found to be deaminated to nicotinic acid which is methylated to trigonelline (Table 7). This observation contradicts the results of Komori & Sendju (1926) who, after feeding nicotinic acid to rabbits, isolated nicotinic acid and nicotinuric acid from the urine, but no trigonelline. The observation is also at variance with the assumption of Huff & Perlzweig (1943a) that rabbits are unable to carry out this methylation. The elimination of known metabolites

is low after ingestion of nicotinamide and nikethamide, but almost complete after the administration of nicotinic acid. The deamination might lead to a thorough disintegration of the pyridine ring. The type of diet seems to have very little influence on the qualitative and quantitative elimination of metabolites except for the increase in 'pseudo-nicotinamide methochloride' after administration of nicotinamide, which occurs only in rabbits fed on oats. The increased elimination of trigonelline following nicotinic acid injection is relatively higher after smaller than after bigger doses and indicates that the methylating mechanism is exhaustible.

The urine passed by rabbits after administration of nikethamide exhibits without any treatment a strong purplish blue fluorescence in the long ultraviolet which increases in intensity with rising nikethamide administration. The urine ceases to be fluorescent about 10 hr after the injection. Some preliminary data of the isolation and properties of the substance responsible for this fluorescence have been obtained. The fluorescent substance is not extractable from neutral, acid or alkaline urine by ethyl acetate, benzene, isobutanol, light petroleum, chloroform or ether and is stable to boiling in neutral, acid or alkaline solution. It is not adsorbed on decalco or charcoal, but is adsorbed on Al_2O_3 (Brockmann) from dry ethanol solution. For its isolation the urine was acidified with HCl, filtered through a decalco column, and shaken with charcoal to remove as many of the other urine constituents as possible. The colourless filtrate was evaporated to dryness, extracted with hot dry ethanol and filtered through an Al_2O_3 column, the fluorescent material being completely adsorbed. The column was thoroughly washed with dry ethanol. Two fluorescent zones were seen on the upper part of the column, a narrow one of purplish blue fluorescence on the extreme top and a wider one of bright blue fluorescence clearly separated from it farther down. The top layer was cut off and both layers were eluted with 50% (v/v) ethanol for the top layer and 85% (v/v) ethanol for the other one. Both eluates were evaporated to a small volume *in vacuo* and treated with non fluorescent acetone. In both cases part of the fluorescent material was precipitated and another part remained dissolved in acetone. The precipitates from the

top layer (A) and from the lower layer (B) were filtered off, washed with dry ethanol and twice recrystallized from hot ethanol. The main fluorescent material (C) dissolved in acetone was brought to dryness by evaporation *in vacuo*, dried *in vacuo* over P_2O_5 and recrystallized three times from hot dry ethanol. The three substances had the following properties.

Substance A m.p. 166.5–168.5°, ultraviolet absorption in water, no band between 260–380 μ , general absorption from 280 to 220 μ with shoulder at 260 μ , $E_{1\text{cm}}^{1\%} = 2.5$.

Substance B m.p. 325° with decomposition and sublimation, ultraviolet absorption in water similar to A with much smaller general absorption.

Substance C very hygroscopic, colourless, turns brown when exposed to air. M.p. 96.5°, if heated to 108.5°, it does not resolidify on cooling, ultraviolet absorption in water distinct band at 336 μ , $E_{1\text{cm}}^{1\%} = 4.6$, with general absorption from 280 to 220 μ , shoulder at 265 μ , $E_{1\text{cm}}^{1\%} = 3.4$ and indications of a peak at 220 μ , $E_{1\text{cm}}^{1\%} = 17.0$. In a cathoretic experiment it formed a uniform band and moved slowly to the anode. It has not been possible so far to collect sufficient quantities of the fluorescent pigments for analysis and estimation of the mol. wt.

Nikethamide is toxic to rabbits and larger doses have to be divided, the total amount which can be administered is, therefore, small. Moreover, the mechanism involved in the production of the fluorescent pigments seemed to be quickly exhausted, the animals used have to rest for a considerable time before they can be used with advantage for a second collection. None of the substances seems to be identical with 1-methyl-2 (or 6) pyridone-3-carbondiethylamides, these differ from the pigments by their fluorescence and ultraviolet absorption. Perhaps the pigments are derivatives of a dihydropyridine which, according to Warburg & Christian (1936), has a band at 340 μ .

Guinea pigs differed from all other species examined by not eliminating any metabolite except 'pseudo-nicotinamide methochloride' (Table 3) and by not methylating any extradietary nicotinamide derivative (Table 8). Slight increase of the 'pseudo-nicotinamide methochloride' occurred after nicotinamide and nikethamide, but not after nicotinic acid administration. Nicotinamide was completely de-

Table 8 Average response to extradietary nicotinamide derivatives by guinea pigs

Compound administered	Dose* (mg)	Metabolites eliminated (% of ingested)*			
		Nicotinamide	Nicotinic acid	Nicotinamide methochloride	Total
Nicotinamide	100	0	32.4	0*	32.4
	200	0	24.2 [2]	0* [2]	24.2
	400	0	38.9 [2]	0* [2]	38.9
Nicotinic acid	100	0	60.5 [2]	0	60.5
	200	0	82.2 [2]	0	82.2
	400	0	66.7 [2]	0	66.7
Nicotindietethylamide	25	12.9	12.7	0†	25.6
	50	10.3	20.4 [2]	0†	30.7

[] indicates time in days over which elimination occurred, if more than one.

Neither trigonelline nor nicotinic acid were eliminated.

* See note to Table 2b.

† Indicates that after administration of nicotinamide or nicotindietethylamide a substance was eliminated to a slightly increased extent giving a positive reaction with the acetone, but not with the König (1904) method.

aminated to nicotinic acid. Of the ingested nicotinamide or nikethamide 25–38 % was eliminated and of the nicotinic acid about 60–80 %

DISCUSSION

The most surprising result is that the two compounds described by other authors (Ackermann, 1913, Komori & Sendju, 1926, Linneweh & Reinwein, 1932*a, b*, Melnick *et al* 1940 and Perlzweig *et al* 1940) as the metabolites of nicotinic acid or nicotinamide in dogs, men or rats, viz trigonelline and nicotinuric acid, are not eliminated by any of these species normally or after administration of the tested compounds. It is easy to understand that authors using the König (1904) method for assay have described the occurrence of trigonelline in the urine before nicotinamide methochloride had been established as a metabolite. The two compounds cannot be distinguished by the König method. However, it has been claimed that these two substances can be isolated from urine and analyses for the compounds or derivatives have been given. Ackermann (1913) e.g. analyzed the gold salt of a substance obtained from urine of dogs after feeding nicotinic acid for gold, carbon and hydrogen, but not for nitrogen, and claimed to have identified it as a salt of trigonelline. The theoretical values of gold, carbon and hydrogen are almost identical for the gold salts of trigonelline and nicotinamide methochloride, while the nitrogen value of the latter is about twice that of the former. The same omission explains the results of Komori & Sendju (1926) who only made an analysis for gold. The results of Linneweh & Reinwein (1932*a, b*), who have produced analytical values for carbon, hydrogen and nitrogen in agreement with trigonelline itself for a substance isolated from human urine, are more difficult to explain. This probably was pure nicotinamide methochloride. With regard to nicotinuric acid, Ackermann's (1913) results on dogs are inexplicable since injected nicotinuric acid is completely split by the dog and eliminated as nicotinamide, nicotinic acid and nicotinamide methochloride (Table 4). It cannot, therefore, be an end product of nicotinic acid metabolism in the dog. This breakdown to nicotinamide, however, explains the anti blacktongue (Woolley, Strong, Madden & Elvehjem, 1938) and antipellagra activities (Elvehjem & Teply, 1943) of nicotinuric acid. The findings of Melnick *et al* (1940) that man eliminates nicotinuric acid after ingestion of doses of 500 mg and more of nicotinic acid have not been tested. It is probable that their method of assay is not suitable for separate determination of nicotinamide and nicotinuric acid.

The contradictory results of the many investigations are mainly due to the faults of the assay methods based on the König principle. The conditions of hydrolysis are of great importance for a clean

separate determination of the metabolites as well as for securing low blanks. Melnick *et al* (1940), Perlzweig *et al* (1940), Sarett *et al* (1942), Sarett (1942) and Huff & Perlzweig (1942) use for the hydrolysis of nicotinamide and nicotinuric acid *N*-hydrochloric acid and 5*N*-hydrochloric acid respectively. The latter produces strongly coloured hydrolysates and, consequently, such high blank values that correct absorption readings are almost impossible. This can be avoided by using *N*-sodium hydroxide for the hydrolysis of nicotinuric acid. Bandier & Hald's (1939) method for the removal of interfering pigments is satisfactory, while Wang & Kodicek's (1943) method is tedious and results in a strong yellow tint interfering with the assay. The modification of the method of Bandier & Hald (1939) by Carter & O'Brien (1945) with regard to pH and amount of metal is advantageous. By systematic study of the effect of pH and amount of metal used on the reproducibility and intensity of colour, the method has been further improved. Contrary to the findings of Bandier & Hald (1939), exposure to daylight, even direct sunlight, does not interfere with the colour intensity.

Considering the metabolism of nicotinamide derivatives, the mammalian species examined so far can be classified into two groups, man, dog, cat and rat which aminate nicotinic acid to nicotinamide, whilst rabbit and guinea pig deaminate nicotinamide to nicotinic acid. Before drawing any further conclusions more species have to be examined, but it is attractive to base on this and similar fundamental functions a classification of species on the basis of their biochemical properties. It has already been mentioned that some mammalian species such as rabbit and horse do not methylate nicotinic acid or nicotinamide whilst other species do so. Whether the type of diet (carnivore, omnivore or herbivore) is responsible for these differences is doubtful. It cannot play any role in the process of methylation since herbivores like rabbit and guinea pig differ in this respect. The mechanisms of methylation and amination of these compounds have been elucidated and their sites located in the liver (Perlzweig, Bernheim & Bernheim, 1943) and kidney and brain (Ellinger, 1946, 1948), respectively. It would be interesting to see whether the methylating enzyme is absent from guinea-pig liver and where and how the deamination occurs in rabbit and guinea pig. It is possible that aminating and deaminating enzymes are present in both types of species, the one being inhibited in normal conditions in the living animal as has been shown for the sulphonamide acylating and deacylating enzymes by Krebs, Sykes & Bartley (1947). The instability of nicotinamide in rabbit and guinea pig suggests that the nicotinamide moiety of the coenzymes might be replaced by nicotinic acid in these species.

The metabolism of nikothlamido particularly in man, rabbit and guinea pig shows, as already suggested by Ellinger *et al* (1947), that this compound is broken down by do othylation to nicotinamide followed, in rabbit and guinea pig, by doamination of the latter compound

SUMMARY

1 The metabolism of nicotinamide derivatives in men, dogs, cats, rats, rabbits and guinea pigs has been studied under normal conditions and after administration of nicotinamide, nicotinic acid, nikethamide and, in some instances, of nicotinamide methochloride and nicotinuric acid. The metabolites tested for have been nicotinamide, nicotinic acid, nicotinuric acid, trigonelline and nicotinamide methochloride.

2 Under normal conditions men eliminate nicotinamide, nicotinic acid and mainly nicotinamide methochloride, dogs and rats nicotinamide and nicotinamide methochloride, cats only the latter compound, rabbits nicotinic acid and guinea pigs none of these compounds. No nicotinuric acid or trigonelline was found in the urine. Rabbits and guinea pigs, however, eliminate small amounts of an unknown substance, giving a positive reaction by the acetone method.

3 Extradietary nicotinamide was eliminated by men almost exclusively as nicotinamide methochloride, by dogs, cats and rats partly as nicotinamide, partly as nicotinamide methochloride, by rabbits as nicotinamide, nicotinic acid and trigonelline and by guinea pigs as nicotinic acid, in the last-named species the unknown compound giving a

positive reaction with the acetone method was slightly augmented.

4 Nicotinic acid was eliminated by men mainly as nicotinamide methochloride and to a very small extent unchanged and as nicotinamide, by dogs, cats and rats in the form of all three metabolites, by rabbits partly unchanged, partly as trigonelline and by guinea pigs unchanged.

5 Nikothlamido is broken down to nicotinamide and accordingly further metabolized by all species. In rabbits after the injection of nikethamide, purplish blue fluorescent pigments occur in the urine, three of which have been isolated.

6 Men, dogs, cats and rats amminate nicotinic acid, rabbits and guinea pigs deaminate nicotinamide, all species except guinea pigs methylate the metabolites to nicotinamide methochloride or trigonelline, respectively, nicotinuric acid is broken down by dogs and rats to nicotinic acid and nicotinamide and cannot, therefore, be an end product of metabolism, while nicotinamide methochloride is eliminated unchanged by rats.

7 DL-Methionine has no constant effect on the metabolism of the various derivatives in rats and is toxic to these animals in larger doses.

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Sulphur Compounds of the Genus *Allium*

DETECTION OF *n*-PROPYLTHIOL IN THE ONION THE FISSION AND METHYLATION OF DIALLYL DISULPHIDE IN CULTURES OF *SCOPULARIOPSIS BREVICAILIS*

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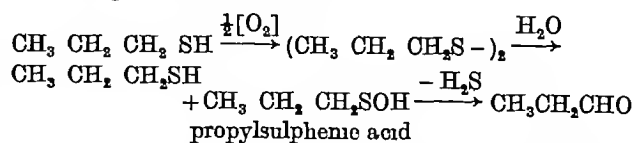
(Received 29 April 1948)

The chief constituent of the essential oils of onion and garlic is usually stated to be allyl sulphide (Whitmore, 1937, Bernthsen & Sudborough, 1931, Vass, 1939). This is an erroneous view arising from a statement by Wertheim (1844) and was first contradicted by Semmler (1892). He found no allyl sulphide, but by distilling the oil of onions, *Allium cepa*, under reduced pressure and collecting various fractions he concluded from the boiling point and other physical properties that the main constituent is a disulphide, $C_6H_{12}S_2$, probably propyl allyl disulphide. The oil amounted to only 0.005% of the whole weight of the onions. Semmler (1892) states that oil of garlic, *A. sativum*, contains 6% propyl allyl disulphide, 60% diallyl disulphide, 20% diallyl trisulphide, and some diallyl tetrasulphide.

Kooper (1910) found thiocyanic acid and also allyl thiocarbimide in freshly expressed, weakly acid onion juice. No formaldehyde, acetaldehyde or acrolein was found. Platenius (1935), Platenius & Knott (1941), Sherratt (1943), Currier (1945) and Dyer, Taylor & Hamence (1941) estimated the total volatile sulphur in onions by conversion to sulphate. Walker, Lindegren & Bachmann (1925) found the fungicidal principles in onion juice to be of two types, one of which is non-volatile and stable to heat. The

other is volatile, and passes off from the extracted juice at room temperature within a few hours. No attempt was made by these later authors to identify the compounds in question.

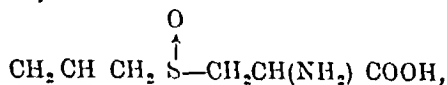
The work of recent investigators. Kohmann (1947) reports the presence of propionaldehyde in onions from evidence based on the melting points and analyses of the 4-nitro- and 2,4-dinitro-phenylhydrazones. He also states that the lachrymatory principle is probably a thioaldehyde, and that sulphur analyses indicate that this may be thiopropionaldehyde. It seems possible that, during the distillation under reduced pressure in presence of water at 50° which Kohmann employed, propionaldehyde might have arisen from propylthiol by the following series of reactions



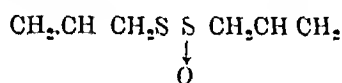
This type of reaction was observed by Schoberl (1933, 1936) with many disulphides under mild alkaline conditions and by Challenger & Rawlings (1937) with diethyl disulphide in a closed copper vessel at 210°.

Kohmann (1947) observed the smell of hydrogen sulphide during the treatment with 2,4-dinitrophenylhydrazine. This might also have arisen from the reaction of the monomolecular thioaldehyde with the phenylhydrazine derivative. However, the percentage of sulphur found by Kohmann is also very close to the figure required by propylthiol or dipropyl disulphide. There is no conclusive evidence that the distillate contained a thioaldehyde.

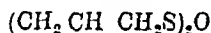
Stoll & Seebeck (1948) have isolated a new amino-acid from garlic, *A. sativum*, and from the crow onion or ramsons, *A. ursinum*, which they name alliin. They have shown this to be L S allylcysteine sulphoxide,



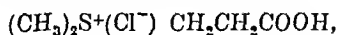
and regard it as the parent substance of the alliin obtained by Cavallito & Bailey (1944) from *A. sativum*. Cavallito, Buck & Suter (1944) and Cavallito, Bailey & Buck (1945) considered that alliin was either



or



The results of Stoll & Seebeck favour the first structure. It was pointed out by Challenger (1946) that, owing to the well-known basic properties of the sulphoxides, alliin may be regarded as of sulphonium type. The importance of sulphonium compounds in nature is emphasized by other recent work. Maw & du Vigneaud (1948) find that the dimethyl β -carboxyethylsulphonium chloride,



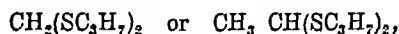
isolated from the marine alga *Polysiphonia fastigiata* by Challenger & Simpson (1947, 1948) and also dimethylcarboxymethylsulphonium chloride possess a labile methyl group and support the growth of rats on a diet deficient in methionine but containing homocystine. A similar observation has been made with the last-named substance by Dubnoff & Borsook (1948). On acetylation of alliin with thioacetic acid Stoll & Seebeck (1948) found that the sulphoxide group is simultaneously reduced to a sulphide link giving the N-acetyl derivative of S-allylcysteine. This and the corresponding S-propyl derivative yield allylthiol and n-propylthiol, respectively, on hydrolysis with sodium hydroxide.

The exact bearing of these observations on our detection of n-propylthiol (see p. 89) and on Kohmann's (1947) results cannot at present be assessed. Alliin has, moreover, not yet been reported in the onion.

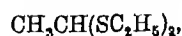
Aspiration experiments with onions. We find that when a slow stream of sterile air is passed over

freshly chopped, peeled onions n-propylthiol volatilizes and can be characterized as mercury di-n-propylthiol by absorption in mercuric cyanide. This was observed with three separate batches of onions, and the thiol was also converted to the silver and lead derivatives.

n-Propylthiol has not till now been detected in nature, although Challenger & Rawlings (1937) observed its formation from di-n-propyl disulphide in cultures of *Scopulariopsis brevicaulis*. Its high volatility probably prevented its detection by Semmler (1892) or other workers, or it might, in their experiments, have been converted to dipropyl disulphide by the oxygen of the air. It may be identical with the very volatile compound mentioned by Walker *et al.* (1925). These authors state, 'it is quite probable that the volatile substances released upon crushing the tissue do not occur thus exactly as they existed in the host cell'. We cannot say whether free n-propylthiol occurs in the undamaged bulb or whether it is liberated by enzyme action after slicing. We considered the possibility that n-propylthiol may not actually be present in the vapours evolved by the onions, but might arise by the interaction of some more complex substance, e.g. a n-propylthioacetal such as



with the mercuric cyanide used as absorbent. Thioacetals $\text{RCH}(\text{SR}')_2$ react with hot aqueous mercuric chloride giving R'SHgCl as shown by Holmberg (1932) and by Taylor (1937), see also Hellstrom & Holmberg (1935). The two propyl derivatives were not available, but we find that diethylthioformal, $\text{CH}_2(\text{SC}_2\text{H}_5)_2$, and diethylthioacetal,



are inert to cold aqueous mercuric cyanide. There seems, therefore, no reason for doubting the presence of free n-propylthiol in the vapours from the sliced onions.

The literature contains no mention of the occurrence of alkylthiols in oil of onions, but Semmler (1887) states that traces of a thiol accompany the divinyl sulphide which he isolated, after treatment with metallic potassium, from the oil of *Allium ursinum*.

Diallyl disulphide and cultures of Scopulariopsis brevicaulis. Challenger & Charlton (1947) have discussed the possible origin of the diallyl disulphide which occurs in oil of garlic (Semmler, 1892). It was, therefore, considered desirable to examine the metabolism of this compound under biological conditions where chemical change could readily be detected. This seemed convenient in view of the investigation of the behaviour of various sulphur compounds in mould cultures, and especially of dimethyl β -carboxyethylsulphonium chloride (recently isolated from the alga *Polysiphonia fastigiata* by Challenger

& Simpson, 1947) which is being made by Mr Y C Liu in these laboratories. Consequently a study of the fate of diallyl disulphide in cultures of the mould *Scopulariopsis brevicaulis* was undertaken.

We find that this undergoes fission with formation of allylthiol which we have characterized as the mercury, lead and silver derivatives. Methylation also occurs and methyl allyl sulphide is produced, this has been characterized as the addition products with mercuric and platonic chlorides. These findings are in agreement with the work of Challenger & Rawlings (1937) and Blackburn & Challenger (1938) who showed that saturated disulphides R_2S_2 , where R is methyl to n amyl, in cultures of *S. brevicaulis*, yield the corresponding alkylthiol RSH and the alkyl methyl sulphide $RSCH_3$. Challenger & Ellis (1935) found that allylarsonic acid in cultures of the mould is converted to dimethylallylarsine thus showing that the allyl group, when attached to arsenic, is not reduced by the mould. Our results with diallyl disulphide show that this is also true when the allyl group is linked to sulphur.

EXPERIMENTAL

Detection of n propylthiol in the onion

Treatment of onions Three separate batches of onions, 1070, 1350 and 1710 g, were peeled, sliced and chopped immediately before use. Air was passed through conc H_2SO_4 , a 1% $HgCl_2$ solution and two tubes of cotton wool (to remove any traces of S compounds and also micro organisms), then through the chopped onions in 0.5 l jars, and finally through various absorbents. Two tubes of aniline removed any thiocarbimide as a thiourea derivative, a tube of 2N HCl eliminated aniline vapour, two tubes of 4% $Hg(CN)_2$ solution removed thiols, and finally two tubes of 3% $HgCl_2$ solution absorbed the sulphides and disulphides which are known (Challenger & Rawlings, 1937, Blackburn & Challenger, 1938) to pass unchanged through $Hg(CN)_2$. The suitability of this procedure was checked by a preliminary experiment in which the constituents of a mixture of allylthiocarbimide and diallyl disulphide were satisfactorily separated.

Identification of n propylthiol Formation of a precipitate in the $Hg(CN)_2$ occurred after 6 hr and ceased in 2–3 days. The m.p. was 63–65° and, after one recrystallization from alcohol, 66–67°. The mixed m.p. with authentic mercury di n propylthiol of m.p. 67° was 67–68°. Mixed m.p. with mercury diallylthiol of m.p. 74° was 60–63° (Found C, 20.8, H, 4.06. Calc. for $C_6H_{14}S_2Hg$ C, 20.5, H, 4.00. Mercury diallylthiol, $C_6H_{10}S_2Hg$, requires C, 20.75, H, 2.90%). The first batch of onions yielded 0.025 g (equivalent to 10 mg of propylthiol/kg).

The second and third batches yielded 0.013 and 0.042 g of mercury di n propylthiol representing 4 and 11 mg of propylthiol/kg. The m.p. and mixed m.p. determinations confirmed the conclusions of the first experiment.

A portion of the mercury compound was decomposed by 6N HCl, the thiol passed through water, and into a 20% solution of lead acetate. The lead salt of the thiol had m.p. 92°. The mixed m.p. with lead di n propylthiol (m.p.

95°) was 94°. Lead diallylthiol melts at 108°. The remaining lead thiol was decomposed with HCl and converted to the silver derivative. Thus, when washed with ethanol and ether had m.p. and mixed m.p. 186° (decomp.) with silver n propylthiol m.p. 186° (decomp.) The corresponding allyl derivative melts at 114°.

Examination of the aniline and of the precipitates in the mercuric chloride Solution of the aniline in 50% (v/v) aqueous acetic acid left a trace of solid indicating the absence of more than traces of a thiocarbimide in the volatile products.

The precipitate in the first $HgCl_2$ tube from three batches of onions was only 0.008 g. It was insoluble in all common solvents, did not melt below 270° and was probably the mercurichloride of an unsaturated sulphide such as



or the mercurated fission product of a disulphide, e.g. $RCH=HgCl-CHOH-CH_2S-HgCl$ (Smith, 1939). Addition of HCl gave the odour of a thiol suggesting that a product of the second type was present.

Preparation of metallic derivatives of authentic allyl and n propyl thiols Lead n propylthiol (Borgstrom, Ellis & Reid, 1929) melts at 94–96°, sintering at 88–89°. The yellow precipitate obtained by us melted at 90–92°. It was recrystallized from benzene, much lead sulphide formed by decomposition by the hot solvent being removed. The m.p. was sharp and constant at 95°. The decomposition of lead dialkylthiols by heat, giving lead sulphide and a dialkyl sulphide, is well known (Klason, 1887, Challenger & Rawlings, 1937). Mercury diallylthiol, $(C_2H_5S)_2Hg$, was prepared by shaking the thiol (2 g) with aqueous 4% $Hg(CN)_2$ solution (700 ml, 4.5 mol) for 2 hr. Reaction was rapid. The sticky, greenish precipitate was separated, washed with water and with ethanol and recrystallized 3 times from ethanol. It formed colourless pearly plates, m.p. 74°. These gradually became pale green in colour (Found C, 20.8, H, 2.8, S, 18.95. $C_6H_{10}S_2Hg$ requires C, 20.75, H, 2.90, S, 18.46%). A specimen which had been kept in a closed tube for 10 years was found to be almost completely black and had an odour of a sulphide.

The lead derivative of allylthiol had m.p. 105–108° and 107–108°, constant, after recrystallizing from benzene. It was again found necessary to remove lead sulphide.

The silver derivatives are insoluble in all common solvents and could not be recrystallized. The allyl derivative melted at 113–114°, darkening above 100°, whereas the n propyl compound melted between 182 and 190° (decomp.) the m.p. varying with the rate of heating, but always lying within these limits. The m.p. were unchanged after washing the compounds with various solvents. The silver derivatives were fairly stable, but the lead derivatives, especially lead diallylthiol, decomposed on keeping for 2 or 3 days.

Preparation and reactions of reference compounds

Preparation of methyl allyl sulphide Methylthiol, prepared from *S*-methylisothiurea sulphate and NaOH (Arndt, 1921), was passed through dilute H_2SO_4 , dried by $CaCl_2$ and absorbed in ice cold sodium ethoxide. The solution of sodium methylthiol was then added in the cold to the theoretical weight of allyl bromide in ethanol under reflux. After refluxing for 30 min the mixture was cooled, poured into water and the oil separated and dried over $CaCl_2$. After four distillations, the b.p. was 91–93°, the

figure given by Obermeyer (1887) (Found C, 54.6, H, 9.45. Calc for C_4H_8S C, 54.6, H, 9.10%) The product was free from thiol, no precipitate being formed on shaking with 4% $Hg(CN)_2$ solution

Derivatives of methyl allyl sulphide The compound formed with excess $HgCl_2$ could not be recrystallized and was infusible. A soluble mercurichloride, m p 115° , was prepared by shaking together equimolecular quantities of methyl allyl sulphide and $HgCl_2$ solution, and filtering immediately. The platinumchloride was prepared from platinum chloride (1.0 g) in water (2–3 ml) and methyl allyl sulphide (0.8 g). The solid was extracted with ether, removal of the solvent left a yellow powder which was recrystallized 5 times from dilute ethanol. The m p (150°) was unchanged by a sixth recrystallization from chloroform-light petroleum. (Found Pt, 38.2 (C_3H_5S CH_3) $_2PtCl_4$ requires Pt, 38.0, C_3H_5S CH_3 $PtCl_4$ requires Pt, 45.8, C_3H_5S CH_3 $PtCl_4 \cdot 2H_2O$ requires Pt 42.2%)

Action of methyl iodide on methyl allyl sulphide Elimination of the allyl group. Equimolecular quantities of methyl allyl sulphide (1.2 g) and methyl iodide (1.73 g) were mixed and left at room temperature for 48 hr. The resulting solid, rubbed with ether and recrystallized from absolute ethanol by addition of ether, had m p 206° . The mixed m p with authentic trimethylsulphonium iodide of the same m p was 206.5° .

Preparation of diallyl disulphide This was prepared by the thiosulphate method of Stutz & Shriner (1933), bearing in mind the ready decomposition of diallyl disulphide in presence of alkali giving, presumably, allylthiol and allylsulphemic acid $C_3H_5S \cdot OH$ (cf. Schöberl, 1933, 1936). By limiting the time of hydrolysis of the sodium allylthio sulphate to 10 min., thus preventing hydrolysis of the diallyl disulphide, and by thorough washing and drying of the disulphide, an almost pure fraction of b p $79^\circ/16$ mm pressure was obtained after three distillations. Von Braun (1903), who prepared it from sodium disulphide and allyl bromide, gives b p $77-82^\circ/15$ mm. (Found C, 49.8, H, 7.2, S, 43.1. Calc for $C_6H_{10}S_2$ C, 49.3, H, 6.9, S, 43.8%) The product may have contained a small amount of the monosulphide. The absence of thiol was shown by shaking with 4% $Hg(CN)_2$ solution, when no precipitate formed.

By alkaline hydrolysis of sodium allyl thiosulphate in the cold for 30 min., separating the oil and boiling the aqueous liquid to complete the reaction, an improved yield of about 30% was obtained.

Action of diallyl disulphide on $PtCl_4$ and $HgCl_2$ When 2 drops of the disulphide and 0.1 g of $PtCl_4$ in a small amount of water were mixed an orange solid formed during 24 hr. Most of this was insoluble in ether, ethanol and chloroform and did not melt below 270° . Ether extracted traces of a solid with similar properties. The compound formed from diallyl disulphide and excess of 3% $HgCl_2$ solution was also insoluble and did not melt below 270° .

Diallyl disulphide and Scopulariopsis brevicaulis in bread cultures

Formation of methyl allyl sulphide and allylthiol Four 1 l. conical flasks, each containing 150 g of breadcrumbs and 25 ml of distilled water, were sterilized at 20 lb pressure (120°) for 25–30 min., inoculated with spores from a potato

agar subculture of *S. brevicaulis* and incubated for 6 days at 32° and for 3 days at room temperature. Diallyl disulphide (0.25 g), emulsified with 25 ml of sterile water, was then added by means of a sterile pipette to each flask. The flasks were connected in series by means of sterile rubber bungs and glass tubing, and sterile air passed through into two tubes of 4% $Hg(CN)_2$, two of $PtCl_4$ and finally two of 3% $HgCl_2$, all in aqueous solutions.

Characterization of allylthiol After 30 min a white solid formed in the first $HgCl_2$ tube, probably due to reaction with unchanged diallyl disulphide, and after 2 hr, a precipitate was observed in the first $Hg(CN)_2$ tube. Next day solid had formed in all absorption vessels except the second $Hg(CN)_2$ tube. A total of 0.20 g (m p 73°) was removed from the first $Hg(CN)_2$ tube (0.12 g after 1 day and 0.08 g after 7 days). After one recrystallization from ethanol, the m p was 74° , unaltered by further recrystallization. The mixed m p with authentic mercury diallylthiol of m p 74° was 74° and with mercury *n*-propylthiol of m p 67° was $56-57^\circ$.

The product was converted to the lead and silver derivatives. The lead diallylthiol melted at $105-106^\circ$ and after one recrystallization from hot benzene, which removed much lead sulphide (see p. 89) had m p $108-109^\circ$ unaltered by further recrystallization. The mixed m p with freshly prepared lead diallylthiol of m p 108° was $108-109^\circ$. The silver derivative after washing with ethanol and ether melted at $111-113^\circ$. The mixed m p with authentic silver allylthiol of m p $114-115^\circ$ was $113-114^\circ$. In each case darkening occurred above $85-90^\circ$. These m p are less sharp than those of the lead and mercury derivatives, but clearly distinguish between *n*-propyl and allyl thiols, as the m p of silver *n*-propylthiol, $C_3H_7S \cdot Ag$, is 186° .

Characterization of methyl allyl sulphide The platinum chloride precipitates were removed after 2 days (0.36 g, m p 118°), after 3 days (0.15 g, m p $123-126^\circ$), after 7 days (0.23 g, m p $126-135^\circ$) and after 15 days (0.14 g, m p $124-136^\circ$). After three crystallizations from aqueous ethanol and two from chloroform-light petroleum ($60-80^\circ$), the constant m p was 150° alone and in admixture with synthetic methyl allyl sulphide platinumchloride of the same m p (Found Pt, 38.04 (C_3H_5S CH_3) $_2PtCl_4$ requires Pt, 38.03%). The unsharp m p of the unrecrystallized products were probably due to the presence of some of the compound formed from diallyl disulphide and platinum chloride. On the nineteenth day 0.025 g of precipitate was removed from the $HgCl_2$ tubes. The amount hardly increased after the second day. It was unmelted below 270° , darkened and sintered above 200° , and was insoluble in all common solvents. Warm dilute HCl gave an odour of a thiol. With NaOH, HgO was precipitated, and only a slight odour of sulphide or disulphide was observed. The results of these tests suggest that the precipitate contained a product of the type $RSHgCl$ or $zRSHgCl \cdot yHgCl_2$, arising from the fission of unchanged diallyl disulphide by $HgCl_2$ (cf. Challenger & Rawlings, 1937) along with a small amount of a mercurio chloride compound of methyl allyl sulphide.

Potassium chloroplatinate, K_2PtCl_6 , was unsuitable as an absorbent for the methyl allyl sulphide, the insoluble product being unmelted below 270° . A second experiment with *S. brevicaulis* and diallyl disulphide gave mercury diallylthiol, m p and mixed m p 74° , and methyl allyl sulphide platinumchloride, m p 149° and mixed m p 150° . The deposit in the $HgCl_2$ solution on extraction with ethanol yielded a

small amount of methyl allyl sulphide mercurichloride, m p 112–114° and 113–114° mixed with an authentic specimen, m p 114° (see p 90) This afforded a second mode of characterization of the mixed sulphide In a third experiment mercury diallylthiol was again obtained

SUMMARY

1 *n*-Propylthiol has been detected in the onion, *Allium cepa*, and characterized as the mercuric, lead and silver salts The thiol was removed from the freshly chopped bulbs in a stream of sterile air and absorbed in mercuric cyanide

2 The behaviour of diallyl disulphide, the main constituent of oil of garlic, *A sativum*, has been investigated in cultures of the mould *Scopulariopsis brevicaulis* which convert it to methyl allyl sulphide and allylthiol These compounds were identified as the platinumchloride and the mercuric, lead and silver salts respectively

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The Effect of L-Glutamic Acid and Other Amino-acids in Hypoglycaemia

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While it is generally agreed that glucose is the only substrate able to maintain the function of the brain in intact animals under normal conditions, opinions are still divided on the part played by certain amino-acids and related substances in cerebral metabolism, particularly in the absence of glucose.

In vitro experiments by Quastel & Wheatley (1932) have shown that L-glutamic acid is oxidized by brain tissue in the absence of glucose. This work has been developed by Krebs (1935) with special reference to glutamine and glutaminase. Weil-Malherbe (1936) suggested that L-glutamic acid fulfils a specific function in relation to carbohydrate metabolism in cerebral tissue.

Using eviscerated animals Bollmann & Mann (1931) and Maddock, Hawkins & Holmes (1939) found that the intravenous injection of the substances known to be oxidized by the brain tissue *in vitro* does not maintain the electrical activity of the brain nor relieve the symptoms of hypoglycaemia. Klein & Olsen (1947) have shown that in cats anaesthetized with sodium amytal substances, such as lactate, pyruvate, L-glutamate and succinate, do not pass from the blood to the brain in significant quantity. Conversely, in the normal human subject a ready exchange of lactate and pyruvate takes place between the blood and the brain as demonstrated by Himwich & Himwich (1946).

The work of Nord (1926) should be mentioned here. We are indebted to Dr R. B. Fisher for drawing our attention to this after a preliminary publication of our results (Mayer-Gross & Walker, 1947). Working with rabbits, Nord produced a rise in the blood glucose by intravenous injection of glutamic and aminoacetic acids, an effect which was abolished after adrenalectomy. He concluded that this effect was due to hypersecretion of adrenalin and subsequent mobilization of glucose.

Further evidence of a direct effect of glutamic acid on cerebral function was provided by clinical observations. Price, Waelsch & Putnam (1943) observed that the administration of DL-glutamic acid hydrochloride reduced the number of *petit mal* attacks in epileptics but was without effect upon major seizures. Waelsch & Price (1944) subsequently showed that this effect was due to the L-glutamic acid fraction and not to any shift in the acid-base

balance of the blood. Zimmermann & Ross (1944) and Zimmermann, Burgemeister & Putnam (1946), confirming these observations, have claimed a favourable influence of orally administered L-glutamic acid upon the ability of rats to learn a simple maze and upon the intellectual development of defective children. At the same time Unna & Howe (1945) have shown that the vomiting induced by the injection of L-glutamic acid is probably central in origin since it can be prevented by narcosis.

While the results of *in vitro* experiments could not be expected to be in complete agreement with these in the intact animal, the wide differences in the experiments quoted above suggested the desirability of a study of the immediate effect upon the human subject of relatively large quantities of L-glutamic acid. The original observation of Quastel & Wheatley (1932) that L-glutamic acid was only oxidized by brain tissue in the absence of glucose suggested that patients in a state of hypoglycaemic coma would prove to be suitable subjects for these experiments.

EXPERIMENTAL AND RESULTS

The subjects of the experiments were otherwise healthy patients undergoing Sakel's insulin coma treatment for schizophrenia. They had, except where otherwise mentioned, received the optimum dose of insulin to produce hypoglycaemic coma. Coma was defined as a state of unconsciousness from which the patient could not be roused by sensory stimulation, if stimulation provoked any movement, it was of a general nature and in no way local or purposeful. The blood glucose levels, estimated by the method of King, Haslewood & Delory (1937), varied in coma between 7 and 12 mg/100 ml, the mean being 10 mg/100 ml. The results of this method closely approximate to true glucose values and we found it reproducible to ± 2 mg/100 ml. At the time of the onset of coma 1 mg of atropine sulphate was injected subcutaneously to minimize the excessive gastric, bronchial and salivary secretion produced by insulin.

Glutamic acid

The results of the oral administration of L-glutamic acid hydrochloride (Waelsch & Price, 1944; Zimmerman & Ross, 1944; Zimmerman *et al.* 1946) suggested that it might be absorbed in sufficient amount from the intestinal canal to serve as a substrate for cerebral metabolism. The substance was first given in hypoglycaemic coma by stomach tube

No effect upon consciousness could be observed in a series of 10 cases in which quantities of up to 100 g of L glutamic acid hydrochloride were administered

In a further series of 14 experiments 20 g of L glutamic acid hydrochloride were added to the 200 g of sucrose normally administered by stomach tube for the purpose of terminating hypoglycaemic coma, in 11 cases no effect upon the recovery of consciousness could be observed. The patients did not wake up more quickly than after glucose alone. The remaining three experiments, however, carried out on the same subject, did suggest that the L glutamic acid hydrochloride might have some effect. This subject had never been known to recover from coma after the oral administration of sucrose. He recovered only after a subsequent intravenous injection of glucose. In these three experiments the addition of 20 g of L glutamic acid hydrochloride to the 200 g of sucrose had the effect of rendering unnecessary the intravenous injection of glucose, consciousness being restored within 20 min of the oral administration of the mixture. Without the 20 g of L glutamic acid hydrochloride intravenous injection of glucose was invariably necessary.

An injectable preparation of L-glutamic acid was prepared by cautious addition of 50% NaOH (w/v) to a saturated solution of L glutamic acid hydrochloride. The two solutions were cooled with ice and mixed with constant stirring in a large mortar packed round with crushed ice. The L glutamic acid at first precipitated was redissolved, and the pH was taken to 7.2 (pH meter). A solution which contained the equivalent of 25 g of L-glutamic acid and 10-11 g of NaCl/100 ml was obtained, and was sterilized by filtration through a Ford 'Sterimat' grade SB.

This preparation (80 ml \equiv 20 g L-glutamic acid) was injected intravenously in a series of 45 experiments carried out on 31 patients in hypoglycaemic coma. The injection was made over a period of 2 min. Blood glucose was determined before the injection and at intervals of 3 min from its termination. The subjects were closely watched for returning consciousness over a period of 15 min, and, if at the end of this period consciousness was not restored, an intravenous injection of glucose was administered. The criteria adopted as signs of consciousness were ability to obey spoken requests and to answer simple questions rationally. In some cases, where speech was delayed, the fact that the patient sat up and swallowed sips of water was considered a sign of consciousness.

In 26 of these 45 experiments the subject became sufficiently conscious to satisfy these criteria while in the remaining 19 cases consciousness was not restored. In many of the latter a considerable decrease in the depth of coma was noticeable, e.g. cessation of hyperventilation and return of normal respiration, disappearance of muscular spasm and twitchings, opening of the eyes, fixation and following the observers' movements, sighing, yawning, stretching, etc.

In all 45 experiments an increase in the blood glucose level was observed. In the 26 subjects recovering consciousness the blood-glucose level at the time of the recovery of consciousness, and in the remaining 19 cases the highest level reached, was noted. These values are set out in columns (1) and (8) of Table 1. The two sets of values are not greatly different from each other, the means being 25 and

22 mg/100 ml respectively. The significance of the rise of blood glucose will be discussed later. There was the possibility that the large quantity of sodium chloride contained in the injected preparation of L glutamic acid could have some effect upon the return of consciousness. For this reason a series of nine identical experiments was carried out in which the comatose subjects were given an intravenous injection of 80 ml of a solution of sodium chloride containing 11 g/100 ml. In no case was there any effect upon the state of consciousness nor any significant effect upon the blood-glucose level, the highest level reached being 16 mg/100 ml while the mean was 13 mg/100 ml. The values are set out in column (10) of Table 1.

Aminoacetic acid

The result of the experiments with L-glutamic acid suggested their extension to other amino acids. Because of its ready availability aminoacetic acid was selected, and an injectable preparation containing 25 g/100 ml was made, the pH being adjusted to 7.2 by means of a few drops of sodium hydroxide solution. The solution was sterilized by intermittent heating to 100° and stored at 37° as the amino acid had a tendency to crystallize at lower temperatures.

A series of 26 experiments was carried out with this preparation under similar conditions to those in the experiments with L-glutamic acid, each subject receiving 20 g of aminoacetic acid. In 13 experiments the patient became sufficiently conscious to satisfy the standard condition while in the remaining experiments this degree of consciousness was not reached although in many cases the coma became considerably less deep. The blood-glucose level at the time of the return of consciousness or, alternatively, the highest level reached in the time of the experiment was noted, these figures are set out in columns (2) and (7) of Table 1. The mean values were 24 and 20 respectively.

p-Aminobenzoic acid

A further series of experiments was carried out using a solution of the sodium salt of p aminobenzoic acid, each subject receiving 20 g of the acid. In four of these six experiments consciousness was restored while the remaining two did not reach this stage. The blood glucose values of this short series are set out in columns (3) and (8) of Table 1, the mean values being 22 and 24 mg/100 ml respectively.

The significance of the rise of blood glucose

The result of these four series of experiments suggested that the injection of 20 g of the amino acid had been responsible for the return to consciousness of the subjects. The mechanism of this result was, however, in some doubt since the administration of

Table 1 *Relationship of blood glucose values and consciousness after the administration of amino-acids, glucose and sodium chloride*

Consciousness restored blood glucose values (mg/100 ml)					Remaining unconscious blood glucose values (mg/100 ml)				
Glutamic acid	Glycine	p Amino benzoic acid	Glucose only, oral	Glucose only, intravenous	Glutamic acid	Glycine	p Amino benzoic acid	Glucose oral and intravenous	NaCl
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
21	23	28	55	53	21	21	23	36	9
16	32	19	54	55	19	14	25	25	12
28	25	23	51	37	26	20	—	35	16
26	23	19	30	32	21	23	—	42	12
23	25	—	37	35	30	26	—	46	14
28	28	—	40	53	25	17	—	23	12
28	19	—	39	48	35	19	—	19	12
32	28	—	35	42	30	19	—	24	12
35	28	—	37	36	17	23	—	22	16
25	21	—	37	37	23	19	—	30	—
34	16	—	34	40	25	27	—	19	—
37	21	—	—	37	32	21	—	34	—
28	19	—	—	—	30	16	—	25	—
23	—	—	—	—	16	—	—	35	—
25	—	—	—	—	16	—	—	19	—
21	—	—	—	—	14	—	—	27	—
35	—	—	—	—	16	—	—	28	—
25	—	—	—	—	16	—	—	27	—
21	—	—	—	—	14	—	—	35	—
27	—	—	—	—	—	—	—	—	—
19	—	—	—	—	—	—	—	—	—
21	—	—	—	—	—	—	—	—	—
19	—	—	—	—	—	—	—	—	—
19	—	—	—	—	—	—	—	—	—
21	—	—	—	—	—	—	—	—	—
21	—	—	—	—	—	—	—	—	—
Mean	25	24	22	42	22	20	24	29	13

the amino-acid had always been accompanied by a rise in the blood-glucose level above the average level of 10 mg/100 ml found in hypoglycaemic coma. The work of Nord (1926) would suggest that the return to consciousness was entirely due to this increase in blood glucose which he proved to be a sympathomimetic effect. On the other hand, Nord had failed to obtain this effect when the quantity of amino-acid injected was less than 2 g/kg, while in none of our experiments did the quantity of amino-acid exceed 0.4 g/kg. Furthermore, no clinical signs of hyperadrenalism such as a rise in pulse rate or in pulse pressure were seen. Previous investigations by ourselves (Mayer-Gross & Walker, 1945) had shown that consciousness was not restored until a blood-glucose level had been reached that was about double the average figures of 22–25 mg/100 ml obtained in these experiments. For this reason it seemed probable that the elevation of blood glucose was not solely responsible for the return of consciousness. It seemed desirable, however, to arrange control experiments on an identical pattern in order to determine the critical level

of blood glucose at which consciousness was restored.

In the first of two series of experiments 200 g of sucrose were administered to subjects in hypoglycaemic coma by a stomach tube. Blood glucose level was determined at 3 min intervals, and the subject closely watched for the appearance of the signs of consciousness satisfying the previously mentioned criteria. The blood glucose level at the time of the recovery of consciousness was noted.

In a second series 3 g of glucose were injected intravenously into patients in coma and similar observations of blood glucose and consciousness were made. Out of a total of 42 experiments consciousness was restored in 23 while in 19 cases the patient remained in coma. The blood glucose levels at which consciousness was restored are set out in columns (4) and (5) of Table 1, the mean values being 42 in each case. In the 19 cases where there was no restoration of consciousness the highest values determined within 15 min after the administration of glucose were noted. These values are set out in column (9) of Table 1 the mean value being 29 mg/100 ml.

Statistical analysis of Table 1 shows that there are clearly no significant differences between the means of columns (1), (2) and (3), nor any significant differences

between those of columns (4) and (5) These columns may, therefore, be taken together The grouped means are, for the amino acids 24.53 and for the glucose, oral and intravenous, 41.34, the difference is 16.81, the standard error of difference is 1.89 and the ratio of the difference to its standard error 8.9 The chance probability of this is less than 0.001 and the difference between the means is highly significant

It may, therefore, be concluded that the rise of blood glucose following injection of amino-acids was insufficient, in itself, to produce the return of consciousness Taking column (9), which gives the blood-glucose values in experiments where consciousness was not restored, the group mean is 29.00, and the difference between this and the grouped mean of columns (1)–(3) is 4.46 The standard error of this difference is 1.96 and the ratio of difference to its error 2.28 The chance probability is less than 0.05 which attains a level of significance commonly accepted for clinical experiments In other words, even in cases in which consciousness was not restored by glucose, the blood-glucose level was significantly higher than that of the cases in which it had been restored by amino-acids

Effect of glutamic acid on the blood urea

An attempt was next made to determine whether the glucose responsible for the rise in blood-glucose level, after the injection of L-glutamic acid, was formed by the synthesis of glucose from the amino-acid If this were so it would be necessary for deamination to occur, and, as a result of this, a rise in the blood-urea level In 12 experiments in which 20 g of L-glutamic acid were injected into patients in hypoglycaemic coma, parallel observations were made upon the blood-glucose level, the blood-urea level and upon the state of consciousness The blood-urea levels were determined by the method of King *et al* (1937) which we found reproducible to ± 1 mg/100 ml The results are summarized in Table 2 In eight cases consciousness was restored at

Table 2 *Parallel observations on consciousness, blood glucose and blood urea after intravenous injection of 20 g of L-glutamic acid in hypoglycaemic coma*

Conscious		Unconscious	
Blood glucose (mg/100 ml.)	Rise or fall in blood urea (mg/100 ml.)	Blood glucose (mg/100 ml.)	Rise or fall in blood urea (mg/100 ml.)
35	+2	23	-3
35	+2	25	-3
28	± 2	25	+5
23	-3	28	+3
21	+6	—	—
32	-1	—	—
25	-5	—	—
25	-2	—	—
Mean 28		25	

an average blood glucose level of 28 mg/100 ml with a slight rise in blood urea in four and a slight depression in the other four Four cases did not recover consciousness, while their mean blood-glucose level rose to 25, in two with a slight rise in the blood-urea level and a slight fall in the remaining two

Succinic acid

Succinic acid is one of the substances related to glutamic acid which may be converted in the body into glucose (Soskin & Levine, 1946) The problem was whether this could take place within the 15 min period of our experiments and affect the hypoglycaemic condition A solution of succinic acid suitable for intravenous injection was prepared by adding small quantities of succinic acid to a solution of sodium succinate until the pH was reduced to 7.2 The quantities were so adjusted that the final product contained the equivalent of 1 g of succinic acid/10 ml

In view of the expectation of very rapid oxidation of the succinic acid by the tissues of the body it was considered necessary to inject the preparation as rapidly as possible An immediate difficulty was encountered, however, in the violent erythema that accompanied the intravenous injection of the substance, and, for this reason, it was deemed inadvisable to carry out the injection too quickly and to administer more than the equivalent of 10 g succinic acid

In a series of 14 experiments no change in the state of consciousness of the hypoglycaemic subject could be seen, nor was there any marked change in the concentration of the blood glucose, the greatest change in the latter being a rise of 5 mg/100 ml

A number of determinations of the blood-succinic acid concentration were made by the method devised by Forssman (1941) The average concentration obtained was 11.6 mg/100 ml the highest being 17.2 mg/100 ml and the lowest 7.3 mg/100 ml The results are summarized in Table 3

Table 3 *The effect of intravenous injection of succinic acid upon blood-glucose and blood-succinic acid concentrations of subjects in hypoglycaemic coma*

(Subjects remained in coma)			Blood succinic acid concentration immediately after injection (mg/100 ml)
No	Succinic acid injected (g)	Rise in blood glucose (mg/100 ml)	
1	2	3	—
2	4	5	—
3	4	3	—
4	8	2	—
5	8	2	—
6	10	2	7.3
7	7	3	10.9
8	12	4	17.2
9	10	4	16.3
10	10	2	11.0
11	10	3	12.2
12	10	5	9.7
13	10	2	8.2
Mean		3	11.6

Individual reactions

Although the amino acid experiments and glucose controls could not always be performed in the same individuals, parallel experiments and controls were possible in 10 subjects. While in some subjects the impression was gained that easy recovery from coma or its persistence after injection of amino acids was peculiar to the individual, critical examination of the experiments as a whole, and of the parallel tests in particular, showed that individual reaction patterns were rare. Success and failure in restoring consciousness seemed to depend mainly on the duration of coma before the administration of the amino acid.

An interesting phenomenon occurred in one female patient who in the normal process of recovery from hypoglycaemic coma after the oral administration of sucrose, invariably passed through a transient but violently emotional phase characterized by much weeping. When she was awakened by L glutamic or aminoacetic acid, the emotional phase did not occur. The patient became completely conscious and able to converse intelligently without emotional upset. Nevertheless, upon her subsequently drinking sucrose solution, the emotional reaction developed. This observation was made in four experiments on successive days.

Glutamic acid in the absence of hypoglycaemia

A series of nine experiments was made in which 20 g of L glutamic acid were injected intravenously to non-hypoglycaemic (fasting) subjects. The clinical condition and the blood glucose level of the patient were closely watched for 20 min after the injection. In spite of administration of atropine, as

Table 4 Blood glucose (mg/100 ml) before and after intravenous injection of 20 g of L glutamic acid to non-hypoglycaemic (fasting) subjects

Before glutamic acid	After glutamic acid				Maximum rise or fall
	5 min	10 min	15 min	20 min	
83	83	83	93	83	+10
65	72	70	65	70	+7
79	67	79	77	79	-12
70	79	77	79	77	+9
101	95	98	88	88	-13
90	97	90	85	99	+9
100	89	72	98	95	-28
97	97	99	90	93	-7
82	80	84	82	82	+2

previously described, the vomitive effect of the L-glutamic acid (Unna & Howe, 1945) was invariably present and was much more marked than in the hypoglycaemic subjects. While there was no detectable effect upon the patient's mental state, there was a generalized sensation of tingling associated with the injection, which had always passed off before vomiting occurred. The effect on blood glucose was very variable, it was raised in four cases, depressed in four cases and not significantly changed in one case. The results which are summarized in

Table 4 suggest that the observations of Quastel & Wheatley (1932) *in vitro* held true *in vivo* and that L glutamic acid was not utilized in the presence of glucose.

DISCUSSION

The work quoted in the introductory remarks suggests two explanations of the restoration of consciousness after the injection of amino acids. Neither accounts for all our observations.

In the first place, the reaction of hypoglycaemic subjects could be due to oxidation of L glutamic acid by the brain in the absence of glucose (Quastel & Wheatley, 1932). Identical reactions obtained with aminoacetic and *p*-aminobenzoic acids make this interpretation doubtful since Krebs (1935) found L glutamic acid to be the only amino acid oxidized by brain tissue.

The second explanation traces the effect of amino acids to the invariable rise in blood glucose. This may be due to (1) general stimulation during injection, (2) conversion of amino acid to glucose, or (3) sympathomimetic action.

(1) While a small rise of blood glucose may be produced in hypoglycaemic coma by any external stimulation, the parallel experiments with 11% sodium chloride solution have shown that this rise is not equal to that produced by amino acids.

(2) From the absence of change in blood urea, after the injection of glutamic acid and from the absence of a rise in blood glucose after injection of succinic acid, it seems improbable that glucose is derived from the amino acid injected. Although it is feasible that glutamic and aminoacetic acids are converted into glucose within the body, this is unlikely in the case of *p*-aminobenzoic acid.

(3) Sympathomimetic action (Nord, 1926), although not observed clinically, cannot be ruled out in our experiments and may cause the rise of blood glucose. Whatever the cause of this rise, however, it cannot account for the recovery of consciousness in a large proportion of cases. Control experiments and statistical analysis of Table 1 have shown that it was too small for this purpose. Furthermore, the injection of glutamic acid into non-hypoglycaemic subjects has completely failed to produce the significant rise of blood glucose demonstrated in Nord's experimental animals. Similarly, the vomiting, much more marked without than with hypoglycaemia, points to a different action of glutamic acid in the two conditions. An explanation based only on the glucose rise therefore fails to account for our observations. Presumably, therefore, the amino acids exert some additional influence on the metabolism of the nerve cell.

The nature of this influence is at present obscure. An excess of simple amino acids may replace fractions of the protein molecule for some of their

functions thus releasing them for a more specific purpose in relation to carbohydrate metabolism. This might result in more efficient utilization of glucose and so in the recovery of consciousness at a lower level of blood glucose

SUMMARY

1 Oral administration of L glutamic acid to patients in hypoglycaemic coma was without effect except in one subject

2 Intravenous injection of 20 g of L-glutamic acid restored consciousness to subjects in hypoglycaemic coma in 26 of 45 experiments, and modified the depth of coma in the remaining 19 experiments

3 Similar effects were produced by the injection of 20 g of aminoacetic and *p*-aminobenzoic acids

4 In all cases where amino-acids were injected

intravenously into hypoglycaemic subjects there was a rise in blood glucose, which was, however, in itself inadequate for the restoration of consciousness

5 Injection of L glutamic acid into subjects in hypoglycaemic coma was without significant effect on blood urea

6 Injection of succinic acid into hypoglycaemic subjects was without significant effect on blood glucose or on state of consciousness

7 Intravenous injection of L-glutamic acid into non-hypoglycaemic subjects failed to produce any significant effect on blood glucose, but produced vomiting far more strongly than in hypoglycaemic subjects

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Studies on the Metabolism of Semen

5 CITRIC ACID IN SEMEN

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The semen of man and certain other higher mammals is distinguished by a very high content of citric acid (Schersten, 1929, 1936, Dickens, 1941, Huggins & Neal, 1942, Lardy & Phillips, 1945, Barron & Huggins, 1946*a, b*, Humphrey & Mann, 1948). The acid originates in the accessory glands of reproduction, chiefly the seminal vesicles, and in this respect it resembles another more recently discovered component of semen, namely fructose,

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which has similarly been shown to be secreted mainly in the seminal vesicles (Mann, 1946). The present study was undertaken primarily with the object of investigating the possibility that there may exist a link between the two substances with regard to their formation, distribution or function in the reproductive organs and semen. In the course of this study it was established that the process of generation and maintenance in semen of both fructose and

citric acid is closely dependent upon and regulated by the same hormone, testosterone. At the same time it was found that, in certain species at least, fructose and citric acid originate in different parts of the reproductive system and that their levels in semen may vary independently of each other. Moreover, in distinction to fructose, citric acid has been found to be metabolized in semen much more slowly than fructose. These and other facts concerning the relation of citric acid to both the anaerobic and aerobic metabolism of semen will be described and discussed in this paper (preliminary communication, Humphrey & Mann, 1948).

METHODS

The material consisted of semen and reproductive organs from several species including ram, bull, boar, stallion, cock, rat and rabbit. The separation of semen into seminal plasma and spermatozoa, and the preparation of washed sperm suspensions were carried out as previously described (Mann, 1945, 1946). Ringer bicarbonate used in experiments to determine the respiratory quotients was made by adding 0.154M NaHCO_3 to the Ringer solution, to give a final bicarbonate concentration of 0.008M, so that when in equilibrium with 95% O_2 and 5% CO_2 the pH of the medium was 7.0. Trichloroacetic acid was employed as deproteinizing reagent for both semen and accessory reproductive organs.

Determinations of O_2 uptake were made in Barcroft differential manometers and in Warburg manometers at 37°. The respiratory quotients were measured in Warburg manometers by the indirect method (Dixon, 1943). Anaerobic experiments were conducted in Thunberg tubes filled with pure N_2 or with 95% N_2 and 5% CO_2 , and the manometric estimation of acid production was carried out in Barcroft differential manometers with gas outlets by measuring the CO_2 output using Ringer bicarbonate and a gas mixture of 95% N_2 and 5% CO_2 . Fructose and fructolysis were determined as described previously (Mann, 1946, 1948). Citric acid was estimated by the method of Pucher, Sherman & Vickery (1936) as modified by Krebs & Eggleston (1944), and the specificity of the method checked according to Breusch & Tulers (1947). Good agreement was obtained between the titrimetric and colorimetric procedures, but the latter was used as a routine. Succinic acid was analyzed according to Krebs, Smyth & Evans (1940), and lactic acid by the method of Friedemann, Cotomo & Shaffer (1929).

RESULTS

Content of citric acid in semen and reproductive organs

Citric acid constitutes a major component of the whole ejaculated semen in several mammalian species including bull, ram, boar, stallion and rabbit. However, with the exception of rabbit, it is usually absent from the epididymal semen, and only small quantities of it are found in ampullar semen. A particularly high concentration of citric acid in whole semen is characteristic for bull where it may

exceed 1%, and also for rabbit and ram. All these three species also show relatively high seminal fructose contents. However, the semen of boar and stallion, which shows relatively low fructose contents, contain much citric acid (130 and 55 mg/100 ml respectively). On the other hand, dog and cock semen appear to be almost entirely devoid of both fructose and citric acid. The species most completely examined was the ram, altogether 48 samples of semen were analyzed during the seven months' breeding season extending from October to May. The lowest value was 66 mg citric acid/100 ml semen, the highest 261 mg/100 ml. The monthly average was higher in October at the beginning of the season (196 mg/100 ml) than late in April when the season was coming to a close (92 mg/100 ml). If two collections of semen were made in quick succession from the same ram, the result was as shown in Table 1. It can be seen that unlike fructose, which was usually higher in the second ejaculate than in the first, citric acid did not always show the same regularity.

Table 1 *Fructose and citric acid in successive ejaculates of ram semen*

Date of collection	Ram (no)	Ejaculate (no)	Fructose (mg/100 ml)	Citric acid (mg/100 ml)
10 Oct	1	1	586	261
		2	800	226
	2	1	484	107
		2	674	178
	3*	1	474	83
		2	536	66
12 Dec	1	1	262	168
		2	364	192
	4	1	328	192
		2	364	144

* This ram had been irradiated with artificial light throughout the previous winter by Dr Yeates and used by him for the study of the effect of light on the reproductive cycle in sheep (Yeates, 1947).

Although both fructose and citric acid are generated in the same part of the reproductive system, the accessory glands, they can be shown to be secreted by functionally and anatomically distinct tissues. The citric acid contents of the various reproductive organs of full-grown animals are given in Table 2. It can be seen that, in rabbit for instance, citric acid is met with principally in the glandula vesicularis rather than in the prostate organ. Yet as previously shown, fructose reaches a higher concentration in the rabbit prostate than in the glandula vesicularis (Davies & Mann, 1947). An even clearer picture was obtained through the study of the accessory glands in the rat which revealed a high concentration of citric acid in the seminal vesicle as well as in the ventral prostate. Yet both these organs are poor in fructose.

(9 and 0 mg /100 g) In the rat, fructose is concentrated mainly in two other organs, namely, the dorso-lateral prostate (82 mg /100 g) and the small gland adjacent to the seminal vesicle proper, known as the 'coagulating gland' or 'anterior prostate' The coagulating gland, thus called because of the presence in it of the semen-coagulating enzyme 'vesiculase', is distinguished by a complete absence of citric acid, at the same time it had a high fructose content (172 mg /100 g)

Table 2 *Distribution of citric acid in male reproductive organs*

Species	Material	Citric acid (mg /100 g tissue)
Boar	Secretion from Cowper's gland	0
	Prostate	38
	Epididymal semen	0
	Secretion from seminal vesicle	580
Bull	Testis	3
	Epididymis	18
	Secretion from the seminal gland	670
	Ampullar semen	550
Rabbit	Epididymal semen	0
	Epididymis	54
	Testis	15
	Glandula vesicularis	84
Rabbit	Secretion of glandula vesicularis	834
	Prostate (I, II and III)	62
	Cowper's gland	42
	Ampulla	273
Rat	Seminal vesicle proper	39
	Coagulating gland	0
	Ampulla	0
	Dorsolateral prostate	20
	Ventral prostate	122

Table 3 *Effect of castration and testosterone treatment on citric acid content of rabbit organs*

	Citric acid in combined tissues of glandula vesicularis, prostate and ampullae (mg /100 g)
Non castrated buck	105
One week after castration	73
Two weeks after castration	22
Five weeks after castration and simultaneous implantation of testosterone propionate (100 mg)	108
Castrated and implanted simultaneously with testosterone, five weeks later pellet removed, rabbit killed after another five weeks	20

Effect of testicular hormone on the formation of citric acid

The level of citric acid in semen and in male reproductive organs is dependent foremost on the degree of sexual maturity of the animal. It is low in young animals which have not yet reached maturity, and is generally linked with the extent of activity of the male sex hormone in the animal body. On castration there is a gradual decline in the citric acid content of accessory glands, and, unless testosterone is applied by injection or implantation, the organs soon become almost depleted of citric acid.

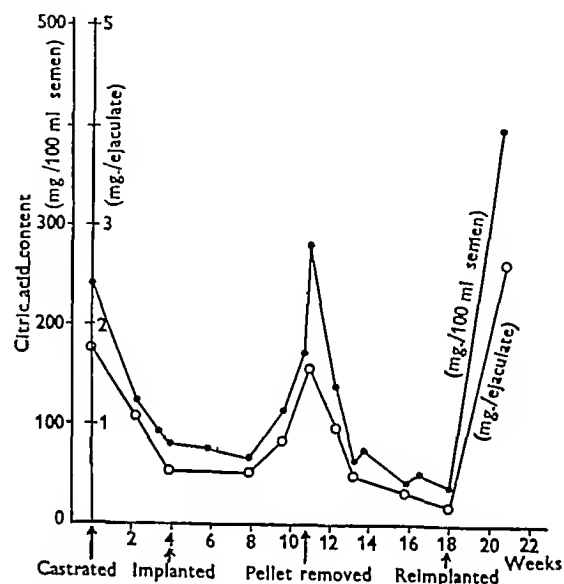


Fig 1 Effect of castration and implantation of testosterone on the citric acid content of rabbit semen. Buck castrated when 18 months old, 4 weeks later implanted subcutaneously with a pellet of testosterone propionate (100 mg), 7 weeks later pellet removed, dried, weighed (80 mg) and preserved, 7 weeks later same pellet reimplanted into the same animal

This can be seen from data recorded in Table 3, which were obtained by analysis of the accessory glands in a series of five full-grown male rabbits. A similar, even clearer, picture of the correlation between citric acid level and testosterone activity was obtained by a direct analysis of the seminal fluid collected by means of the artificial vagina. By this means it was possible to follow closely the sequence of changes brought about by castration and hormone implantation in the intact animal. Fig 1 illustrates the effect of castration and of subsequent implantation of testosterone on the level of citric acid in the semen of the same rabbit, one curve shows the changes in the concentration of citric acid in semen (mg /100 ml) and the other the changes in the absolute quantity of citric acid as represented by a whole single ejaculate. Both curves show clearly the post-castration fall of citric acid in semen and the

recovery which followed implantation of testosterone. A point which deserves special attention is that, as a result of the prolonged treatment with testosterone, the formation of citric acid in the castrated buck has been raised to a level beyond that usually observed in a non-castrated and untreated animal.

Citricolysis

Citricolysis, by which we mean the metabolic breakdown and disappearance of citric acid, was studied (a) in whole semen, by incubating it *in vitro* and allowing it to use up the preformed citric acid, and (b) in washed sperm suspensions incubated with added citric acid. Unlike fructolysis, which proceeds in spermatozoa at a constant and characteristic rate and which can be conveniently expressed in terms of a well-defined 'fructolysis index' (Mann, 1948), the rate of utilization of citric acid was not constant enough to warrant the introduction of a definite 'index'. In any case, in whole semen the rate of citric acid disappearance was found to be much smaller than that of fructose. In bull and ram semen, for instance, the rate of citric acid disappearance was frequently as low as 0.05 mg/hr at 37° in the presence of 10^9 sperm cells, as against 1.5–2.0 mg fructose used up under identical conditions. Occasionally, the rate of citricolysis was higher. This is shown by the experiments recorded in Table 4 in which whole ram semen was used (4×10^9 sperm, and 1.3 mg citric acid/ml), as well as washed spermatozoa of the same concentration, the decrease in citric acid resulting from incubation for 2 hr at 37° is expressed in Table 4 as a percentage of that initially present. It may be added here that, although in whole semen as ejaculated, citric acid appears to be distributed in both the cells and the plasma, the spermatozoa can be easily freed from citric acid by washing with Ringer solution. For instance, the suspension of washed spermatozoa as used in the experiment referred to above was prepared by diluting ram semen with 3 vol of Ringer solution, centrifuging and washing the cells with 2 vol of Ringer. The washed cells were found to be free from citric acid.

Table 4 *Citricolysis in ram spermatozoa*

	Percentage decrease in citric acid content as result of 2 hr incubation at 37°		
	In air	In O ₂	In N ₂
Whole semen	70	73	60
Whole semen diluted with 2 vol Ringer phosphate	40	31	35
Washed sperm suspension (4×10^9 cells/ml.) with 0.1% citric acid and 0.2% fructose added	30	34	33

The process of citricolysis, although rather sluggish, may continue in semen for some time after the spermatozoa have exhausted the entire reserve of seminal fructose, i.e. after fructolysis has come to an end. This was noted both in aerobically and anaerobically incubated semen. Active citricolysis was found to be linked with the presence of sperm cells in semen, the seminal plasma itself being unable to metabolize citric acid. Indeed seminal plasma contains a heat-labile factor which generally inhibited the oxidation of citrate by animal tissues. This inhibitory effect was particularly noticeable when the seminal plasma was added to a liver pulp which alone utilized citrate very efficiently. This is illustrated by the following experiment. Rat liver was ground with 9 vol 0.1M-phosphate buffer, pH 7.4, and three 2 ml samples of tissue pulp were shaken aerobically for 90 min at 37°, with the following additions: (i) Ringer solution (0.8 ml), (ii) Ringer solution (0.8 ml) containing citrate (5 mg citric acid), and (iii) seminal plasma (0.8 ml with a content of 5 mg citric acid). The results (Table 5) show that the seminal plasma had a pronounced inhibitory effect on both O₂ uptake and citrate utilization by the ground liver tissue, dialyzed, but not heated, seminal plasma, caused a similar inhibition.

Table 5 *Effect of bull seminal plasma on the O₂ uptake and citrate utilization by liver pulp*

Additions to liver pulp	Results of 90 min incubation	
	O ₂ uptake (μl)	Decrease in citric acid (%)
Ringer solution	750	—
Ringer with added citrate*	990	87
Seminal plasma	120	5

* Equal to the amount present in the seminal plasma.

However, unlike the citrate oxidation in liver pulp, that in intact cells such as the spermatozoa was not interfered with by the seminal plasma, and proceeded at approximately the same slow rate in sperm suspensions as in whole semen.

An attempt was made to identify succinic acid as a possible intermediary product of the aerobic metabolism of citric acid in spermatozoa. Samples (2 ml) of washed sperm suspensions (2×10^9 ram sperm/ml Ringer-phosphate) were incubated aerobically for 3 hr in Barcroft manometers, in presence and absence of citrate, with and without the addition of 0.005M-malonate. No accumulation of succinic acid was observed in any of these samples (Table 6). It may also be added that the level of succinic acid in fresh bull and ram seminal plasma was determined, it was found to be rather low (5–10 mg/100 ml plasma).

Table 6 *Effect of malonate on aerobic metabolism of citrate by ram spermatozoa*

Additions to the sperm suspension	Changes resulting from 3 hr incubation		
	O ₂ uptake (μl)	Citric acid disappearance (mg)	Succinic acid formation (mg)
None	1040	—	—
Citric acid (2.8 mg)	1045	0.41	0
Citric acid (2.8 mg) plus malonate (0.005 M)	1190	0.57	0
Malonate (0.005 M)	1170	—	0

Citric acid in relation to respiration and fructolysis in semen

Under conditions so far studied, no appreciable effect of citric acid on either fructolysis or respiration of washed sperm suspensions could be detected. This is borne out by the experiment (cf Table 7) which was carried out with a suspension of washed ram spermatozoa in Ringer-phosphate, 0.7×10^9 cells/ml.

The suspension was divided into two parts of which one (A) was treated with 480 mg fructose/100 ml, and the other (B) with 100 mg fructose/100 ml. Each of the two suspensions was then divided into two parts, one of which was left without any further treatment, whilst the other received 180 mg citric acid (neutralized)/100 ml. All four samples were incubated at 37° in narrow test tubes (0.7 cm in diameter, as used for storage of semen required for artificial insemination) and the disappearance of fructose was followed at 30 min intervals.

No significant difference in the rate of fructose disappearance between sperm suspensions containing citrate and those incubated in absence of citrate was observed (cf Table 7). A similar experiment was

Table 7 *Effect of citrate on fructolysis in washed ram spermatozoa*

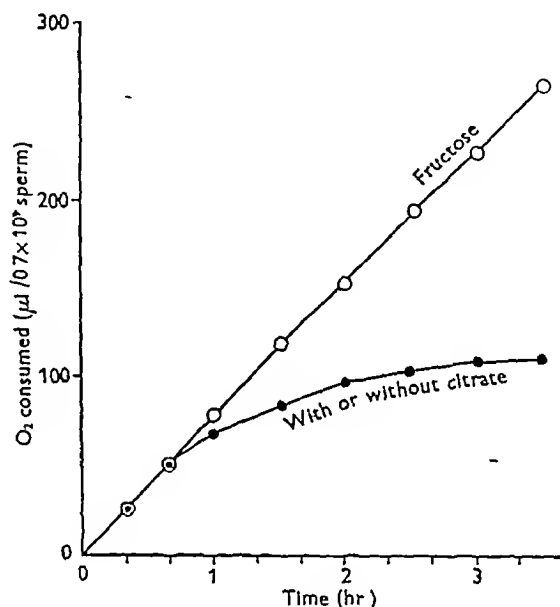
Incubation (min)	Fructose concentration in sperm suspension (mg/100 ml.)			
	Suspension A*		Suspension B	
	Without citrate	With citrate	Without citrate	With citrate
0	480	480	100	100
30	414	415	62	61
60	361	346	27	27
90	294	304	15	12

* Suspensions A and B contained different amounts of added fructose (see text)

carried out with the four sperm suspensions in Ringer bicarbonate instead of Ringer-phosphate and using Barcroft manometers filled with 5% CO₂ and 95% N₂, instead of the narrow-bore tubes employed above. This made it possible to follow fructolysis by manometric measurement of acid production in addition to the chemical estimations of

fructose and lactic acid. Again, no difference could be detected in the rate of fructose disappearance between citrate-free and citrate-containing suspensions, and both produced lactic acid at the same rate. No lactic acid, however, was produced by spermatozoa from citrate alone, i.e. when addition of fructose was omitted.

Previous investigations (Mann & Lutwak-Mann, 1948) have shown that the O₂ uptake of dilute suspensions of washed spermatozoa declined gradually unless the sperm cells were provided with an extracellular source of oxidizable material such as a glycolyzable sugar or lactic acid. In the present study,

Fig 2 *Effect of fructose and citric acid on the respiration of washed ram spermatozoa*

observations were extended to a number of other compounds and in particular to organic acids. Several organic acid salts such as acetate, propionate, butyrate, oxaloacetate and pyruvate, were found to have a pronounced beneficial influence on the respiration of spermatozoa, in accordance with the view held by Lardy & Phillips (1944, 1945). However, the action of these organic acids as revealed by our study was not on the initial rate of respiration and did not result in any marked rise of the initial O₂ uptake. Instead, these substances were found to maintain and prolong the initial rate of respiration, i.e. to delay the decline in O₂ consumption which would otherwise take place. Unlike the glycolyzable sugars and the above-mentioned organic acids, citric acid was found to be unable to maintain the rate of O₂ uptake of ram spermatozoa (Fig 2). In this respect it resembled lactose, ethanol, glycerol, glycine, succinic acid and malic acid, none of which exhibited any marked 'initial rate preserving effect' on the sperm respiration.

The possibility was also examined that citrate may have a stimulating effect on the RQ of spermatozoa. However, unlike fructose, the addition of citrate had, if anything, a slight depressing effect on the RQ of washed ram sperm.

In the course of this study the question was also taken up of possible variations in the respiratory activity of spermatozoa induced by changes in O_2 - and CO_2 -tension. It was found that the value of Z_{O_2} (μl O_2 taken up by 10^8 sperm cells in 1 hr) remained unaltered when the O_2 tension was lowered from 100 to 4%. On the other hand, the sperm respiration was higher in the presence of 5% CO_2 and 95% O_2 than in either air or pure O_2 . The RQ and Z_{O_2} values shown in Table 8 were all obtained using washed sperm suspensions with a final concentration of 2.5×10^8 ram spermatozoa/ml.

Table 8 RQ and Z_{O_2} of washed suspensions of ram sperm

Duration (hr)	No additional substrate	In presence of 0.01 M-citrate RQ	In presence of 0.01 M-fructose
1	0.92	0.83	1.00
2	0.78	0.75	0.91
3	0.84	0.75	0.79
Gas Z_{O_2} at the end of 1 hr			
In pure O_2	11.6	12.0	14.0
In 95% O_2 -5% CO_2	15.6	14.0	19.2

Formation of citric acid in spermatozoa and reproductive organs

In the course of their study on fat and carbohydrate oxidation in mammalian spermatozoa, Lardy & Phillips (1945) demonstrated the presence of aconitase in bull sperm as well as the ability of sperm cells to form citric acid from added pyruvate or oxaloacetate. Using washed ram spermatozoa we were able to confirm these observations. The analysis of citric acid synthesis convinced us, however, that the quantitative contribution of spermatozoa, as regards the final content of citric acid in semen, was very slight or negligible, and that the bulk of the citric acid must be derived from the secretions of the accessory glands rather than from the spermatozoa. In our experience there was no difficulty in completely freeing bull and ram spermatozoa from citric acid by washing them once or twice with Ringer solution. We were also unable to detect any appreciable formation of citric acid on incubation of the washed spermatozoa. In order to induce the sperm cells to form citric acid, a large concentration of added oxaloacetate was found to be necessary. The formation of citric acid from oxaloacetate by washed suspensions of ram spermatozoa is illustrated in Table 9.

In this experiment 4 ml ram semen were diluted with 16 ml Ringer solution, centrifuged, and the sperm suspended in sufficient Ringer solution to give a final volume of 8 ml; this reduced the concentration of preformed citric acid to 0.08 mg/ml. Part of the suspension was diluted with an equal volume of 0.1 M Na oxaloacetate in Ringer solution and kept for 1 hr at 37°. Another sample of the sperm suspension was first inactivated by heating for 2 min in boiling water and then mixed and incubated with oxaloacetate, serving as a control experiment for the non enzymic conversion of oxaloacetate to citric acid.

From Table 9 it can be seen that under the conditions studied no citric acid was formed in the heat inactivated sample. However, the unheated sperm cells produced citric acid from oxaloacetic acid, and the synthesis was greater under aerobic than an aerobic conditions. Aerobically, as much as 0.21 mg citric acid was produced in 1 hr by 10^8 ram spermatozoa. Assuming that the dry weight of 10^8 sperm is 30 mg we arrive at the figure of 0.7 mg citric acid produced by 100 mg sperm, dry weight.

Table 9 Formation of citric acid from oxaloacetic acid by washed suspensions of ram spermatozoa

Pretreatment of sperm	Incubation in	Citric acid content (mg/ml)	Citric acid formed	
			(mg/ml)	(mg/10 ⁸ sperm)
Heat inactivation	N_2	0.06	—	—
None	N_2	0.18	0.12	0.07
Heat inactivation	Air	0.08	—	—
None	Air	0.42	0.36	0.21

Table 10 Anaerobic formation of citric acid from oxaloacetate by rat seminal vesicle and testis

	Citric acid formed (mg/g tissue)
Seminal vesicle	
Heat-inactivated pulp	0.00
Fresh pulp	0.12
Testis	
Heat-inactivated pulp	0.00
Fresh pulp	1.32

A similar study of the citrate synthesis from oxaloacetate was also made with two rat organs, the testis and the seminal vesicle.

The tissues were ground with 5 parts 0.1 M phosphate buffer, pH 7.3, and the suspensions diluted with an equal volume of Ringer solution containing 6.5 mg glucose, 75 mg oxaloacetic acid (Na salt) and 8.5 mg Na pyruvate/ml. The mixtures were then incubated in Thunberg tubes filled with N_2 , and citric acid determined after 1 hr at 37°. Control experiments were run simultaneously with mixtures containing heat-inactivated tissue suspensions.

The analytical results (Table 10) showed that under these conditions the rat testis was more than

ten times as active as seminal vesicle in synthesizing citric acid from the added keto acids. Similar results were obtained with aerobically incubated tissue suspensions, but less significance can be attached to the aerobic experiments in view of the slow, but nevertheless definite, disappearance of citric acid from air-incubated suspensions of ground seminal vesicles.

Regarding the content of aconitase in semen, this was studied separately in washed sperm suspensions and in seminal plasma.

Undiluted whole ram semen was centrifuged, the seminal plasma separated, and the cells washed twice with Ringer solution. The plasma and the centrifuged sperm were then separately diluted with Ringer phosphate up to the original volume of whole semen. To 0.1 ml samples of 'plasma' or 'sperm', 0.3 ml Ringer phosphate and 0.4 ml 0.13M-phosphate buffer, pH 7.4, containing 3 mg *cis*-aconitic acid (Na salt) were added, and the mixtures were kept for 30 min at 30° in Thunberg tubes filled with N₂.

The results (Table 11) showed that the seminal plasma was devoid of aconitase activity. The ram spermatozoa, on the other hand, showed themselves capable of efficiently converting *cis*-aconitic into citric acid, the citric acid formation, expressed in terms of Q_{citrate} (μl citric acid formed in 1 hr by 1 mg tissue dry wt) being 10.7.

Table 11 *Aconitase activity in spermatozoa and seminal plasma*

	Incubation (min)	" Citric acid formed (mg)	Aconitic acid con- verted to citric acid (%)
Sperm suspension	30	1.39	42
Seminal plasma	30	0.00	—

To enable some comparison between the aconitase activity of spermatozoa and that of other animal tissues to be made, two rat organs, liver and seminal vesicle, were assayed for their aconitase content. The tissues were thoroughly ground with 5 parts 0.1M-phosphate buffer and centrifuged, 1 ml extract of 1:50 diluted liver extract and 1 ml of 1:10 seminal vesicle extract were each treated with *cis*-aconitate and incubated as described above for seminal plasma and spermatozoa. The values for Q_{citrate} were 17.2 for rat liver, and 3.6 for rat seminal vesicle.

DISCUSSION

The seminal plasma, i.e. the composite mixture of secretions from the male accessory glands, serves both as a vehicle and nutrient for spermatozoa in whole ejaculated semen, chemically it differs from most other body fluids in several respects. The seminal plasma is remarkable for its high content of three chemical substances, citric acid (Schersten, 1929), fructose (Mann, 1946) and phosphorylcholine

(Lundquist, 1946), all of which appear to originate in the same accessory organ, the seminal vesicle. In this respect, however, the rule is not without exceptions, as shown by our study of rabbit and rat generative organs. In the rabbit, citric acid was met with principally in the glandula vesicularis, whereas the highest concentration of fructose was found in the prostate. In the rat, citric acid was located in the ventral prostate and the seminal vesicle proper, but fructose in the dorsal prostate and in the coagulating gland. Furthermore, it was found that a high concentration in semen of one component does not necessarily run parallel with a correspondingly high level of the other. Boar and stallion semen, for instance, although rich in citric acid, were shown to be comparatively poor in fructose. Moreover, there are daily individual fluctuations in the level of citric acid and fructose in semen, and they were shown not to coincide with each other, also a variable ratio citric acid/fructose was encountered and found to be characteristic of semen. On the whole it seems probable that citric acid of semen is secreted independently of fructose, in the sense that these two seminal components are produced by two distinct types of secretory cells. In one respect, however, the processes of citric acid and fructose generation in the male accessory organs closely resemble each other. As demonstrated in this study, both citric acid and fructose are formed under the influence of, and in close dependence upon, the male sex hormone. Thus the behaviour of citric acid is analogous to that previously described for fructose (Mann & Parsons, 1947), except that the post-castration disappearance and the hormone-induced reappearance of citric acid in seminal plasma are not as prompt as in the case of fructose.

Fructose in semen represents a source of readily available energy for the spermatozoa (Mann, 1946, 1948, Mann & Lutwak-Mann, 1948). With regard to citric acid, however, so far its function is rather obscure and the clarification of this problem must await further study. It is conceivable that citric acid may be associated with the phenomenon of spontaneous gelification, coagulation and subsequent liquefaction which normally occur in the semen of certain species. In this connexion one may recall the finding of Huggins & Neal (1942) that citrate in human semen is the cause of prolonged coagulation time of mixtures of blood and seminal fluid, and that this delay of clotting can be effectively counteracted by adding calcium ions. The possibility of citrate acting as a binding substance for calcium has been envisaged by Schersten (1936) and Huggins (1945), and the fact pointed out that milk, another fluid rich in citrate, has also a high calcium content. In the same connexion our observations may be recalled that in the rabbit citric acid occurs mainly in the glandula vesicularis, that is, in the organ which is

associated with the process of semen gelification. Similarly, in the rat, a high concentration of citric acid was found in the seminal vesicle proper, the organ which normally provides the substrate for vesiculase leading to gel formation.

Hyaluronidase is another enzyme to be considered in connexion with the possible role of citric acid in semen. We carried out some experiments and found that hyaluronidase activity of washed ram spermatozoa was increased by citrate to the same extent as by an equimolar solution of sodium chloride. However, it is possible that the activity of this enzyme is linked with citrate in a more indirect manner, such as that indicated by Baumberger & Fried (1948), who claim that citrate exerts a 'protective action' against antinvasin *in vitro*.

There is no indication that citric acid in semen acts as a major source of nutrient material for the spermatozoa. The rate, both aerobically and anaerobically, at which citric acid is broken down by spermatozoa, whether in whole fresh semen or when added to sperm suspensions, is slight in comparison with the rate of normal fructolysis. When both fructose and citrate were supplied to washed spermatozoa, citrate failed to exert a sparing effect on fructose, and fructolysis went on at exactly the same rate in presence and absence of citrate. Citrate unlike glucose, lactate, pyruvate, oxaloacetate or acetate, was ineffective in maintaining the rate of respiration of washed spermatozoa. On the other hand, however, citricolysis, although slow, continued even after the spermatozoa had used up all available fructose and this, together with the earlier finding of Lardy & Phillips (1945) that citrate has some beneficial effect on the sperm motility, suggests that citrate may be of some specific value to sperm metabolism.

The spermatozoa of the ram contain aconitase, ($Q_{\text{citrate}} = 10.7$), and they are able to form citric acid from added oxaloacetic acid. However, the bulk of citric acid present in whole ejaculated semen is derived from the seminal plasma and not from the spermatozoa. It is interesting to note that under the experimental conditions employed, the rat seminal vesicle, which normally contains more citric acid than, e.g., liver, testis or spermatozoa, was found to have less aconitase activity than these tissues and little ability to form citrate from oxaloacetate. In this respect, the rat seminal vesicle differs from the human prostate which is the chief citric acid producing organ in man, and which, according to Barron & Huggins (1946*a, b*), is particularly rich in aconitase. Before any general conclusions can be drawn concerning the mechanism of citrate production in the male organs of reproduction, it would be essential to extend the investigations to several more animal species. It is also necessary to bear in mind the possibility that accessory glands of re-

production may contain factors similar to that found in bull seminal plasma which was shown to interfere with the metabolism of citric acid in liver pulp.

SUMMARY

1 Citric acid is a major component of semen and the concentration is particularly high in bull semen where it may exceed 1%. It is usually absent from epididymal semen but is present in ejaculated and sometimes also in ampullar semen. It is derived mainly from the secretions of the accessory glands of reproduction, chiefly from the seminal vesicle but also from the prostate.

2 There is no definite correlation between the processes of fructose and citric acid formation in the accessory organs. In the rat and rabbit the two substances are found in two different types of secretory organs.

3 Following castration seminal citric acid disappears, but it reappears in response to testosterone. However, the post-castration fall of citric acid as well as its reappearance after testosterone treatment are less prompt than in the case of fructose.

4 When fresh semen is incubated *in vitro* citric acid is metabolized by spermatozoa both aerobically and anaerobically. The rate of citrate utilization, however, is much smaller than that of fructose. Similarly, washed spermatozoa utilize added citrate much more slowly than added fructose. Seminal plasma itself is unable to metabolize citric acid. It contains a heat-labile factor which inhibits O_2 uptake and citrate oxidation in liver pulp.

5 Citric acid has no effect on the course of fructolysis by washed ram spermatozoa. It is incapable of maintaining the sperm respiration, in this respect it differs from fructose as well as from other organic acids such as lactic, pyruvic, oxaloacetic, acetic, propionic and butyric, all of which prolong the respiration of washed spermatozoa. The RQ of spermatozoa is little affected by citrate, but is increased to 1 by fructose.

6 Ram spermatozoa contain aconitase and are able to form citrate from added oxaloacetate. However, the bulk of citric acid present in whole ejaculated semen is derived from the seminal plasma and not from the spermatozoa. The seminal plasma is devoid of aconitase activity.

7 Rat seminal vesicles, despite their high content of citric acid, appear to have comparatively little enzymic activity associated with the formation of citrate from oxaloacetate.

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A Note on the Disturbance of the Haemoglobin Metabolism of the Rat by Sulphanilamide

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Certain disturbances of haemoglobin metabolism were among the side reactions observed after the introduction of sulphonamide therapy. Animal experiments clearly demonstrated that continued dosage produced an anaemia. In human subjects cyanosis was common, due mainly to methaemoglobinaemia and occasionally to sulphaemoglobinaemia. Drug rashes were also observed in a proportion of cases, and, while not all of the rashes were associated with light sensitivity, the excretion of urinary porphyrin after dosage was found to be increased in a number of cases (cf Rimington & Hemmings, 1938). The excretion of porphyrin in the urine of the rat was then found to be increased many times by dosage with sulphonamides (Wien, 1938, Rimington & Hemmings, 1938, 1939). Other aromatic amino compounds were found to produce a porphyrinuria. Rimington & Hemmings (1939) observed a correlation between the severity of the porphyrinuria and the presence of methaemoglobin in the blood of their animals. Since the porphyrin was found to be coproporphyrin type III, differing only from the protoporphyrin IX found in haemoglobin

in having propionic acid side chains in place of vinyl side chains, they put forward the tentative hypothesis that the coproporphyrin was derived from the increased haemoglobin breakdown under these conditions. A similar view was entertained by Brownlee (1939) as a result of his investigation of the effects following the administration of antipyretics to rats. The appearance of coproporphyrin would signify, according to this hypothesis, a departure from the normal pathway of haemoglobin catabolism.

The present work was begun in order to see if this porphyrinuric action of the sulphonamides in rats could be mitigated by the administration at the same time of substances, such as ascorbic acid, hydrochloric acid, etc., which had been claimed to diminish the severity of the side reactions following sulphonamide therapy. In addition, it was felt desirable to investigate quantitatively the bile-pigment excretion during dosage with the drug so that an estimate might be made of the importance of the normal pathway of haemoglobin catabolism under these conditions. Rimington & Hemmings (1938) had indeed commented on the large amounts of urobilin which they observed in the course of their analyses of faecal porphyrins.

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It was not found possible, however, in our experiments, to repeat the basic observations of Wien (1938) and of Rimington & Hommings (1938), i.e. increased excretion of coproporphyrin after dosage with the drug, and in consequence the immediate clinical importance of the programme was greatly diminished. Transfer of the author to other work has prevented the carrying out of further experiments to elucidate the cause of the disagreement. However, attention has not been drawn to the problem since the completion of the experimental work outlined here (1942) and since additional disturbances of pigment metabolism were noted, the data are presented here in the hope that further interest may be aroused. The positive findings of several individual groups of workers would suggest that the cause of our negative findings might be due to some factor, such as the dietary, and the elucidation of this point would be of no little interest.

EXPERIMENTAL

Adult white rats, 200–300 g in weight, were used.

Diet. Two diets were used. *A*, ground wheat meal 86%, dried whole milk 7%, meat meal 3.5%, bone meal 3.5%; *B*, wheat meal 60%, dried whole milk 10%, meat meal 25%, bone meal 5%. Diet *A* was the stock diet of the rat colony. The animals remained healthy on this diet and reproduced normally. During dosage with sulphanilamide, however, a higher mortality was observed than other workers had reported. Since Smith, Lillie & Sholman (1941) had observed that rats on a high protein diet showed no decline in haemoglobin when dosed with sulphanilamide, and exhibited a lower mortality than when on a low-protein diet, an experiment was carried out with diet *B*.

During an experiment the animals were given water *ad lib*, and were given free access to the diet for 1 hr/day. They remained healthy under these conditions when not dosed with the drug.

Collection of samples. Twelve individual metabolism cages were used. They were supported on waxed aluminum funnels and delivered the urine and faeces on to bulbs, sitting in the necks of 100 ml flasks. The urine was collected in the flask which contained a few drops of toluene, the faeces in a tin surrounding the flask. Mechanical losses of urine amounted to no more than 5% of the total volume. Analyses were carried out on 3 day collections of the urine and faeces.

Administration of drug. Solutions or aqueous suspensions of the drugs used were delivered directly into the oesophagus of the animals with a blunt pipette after their daily feed.

Analytical methods

Urine coproporphyrin. Bulbs and flasks were inspected daily so that urine contaminated with fragments of faeces might be discarded, the samples were then filtered through glass wool and the coproporphyrin taken into ether by adding one third volume of acetic acid and shaking with three portions of solvent. The ether was washed once with a solution of sodium acetate, five times with distilled water and the porphyrin extracted with three portions of approx 0.14N HCl. The concentration of porphyrin in the extract

was determined by matching its fluorescence in filtered ultraviolet light with the nearest of a series of dilutions of mesoporphyrin IX in approx 0.14N-HCl. A red filter, transmitting beyond 630 m μ , excluded the whitish fluorescence of an interfering substance not removed by the preliminary purification. A spectrophotometric check showed that the error involved by using a different porphyrin for comparison could be neglected.

Faecal porphyrin. Ground faeces were left covered with glacial acetic acid for 30 min. They were then thrice extracted with an equal volume of ether. The acid ether extract was then shaken with about 15 ml sodium acetate and with successive portions of water until only traces of urobilin were present in the extract. The sodium acetate and the water washings were retained for the urobilin estimation. Both copro and proto porphyrin were then removed from the washed ether by three extractions with approx 0.14N-HCl. The combined extracts were filtered and the concentration of the porphyrin determined by measurement at 552 m μ in a Hilger Nutting Barfit spectrophotometer.

The total porphyrin was measured in this way because it was not found possible to get a good separation of the two porphyrins by acid fractionation of the crude extract of faeces. The measurement is thus a rather crude index of changes in the excretion of coproporphyrin. At the end of a given experimental period the pooled extracts were further purified and the copro and proto porphyrins extracted and determined separately.

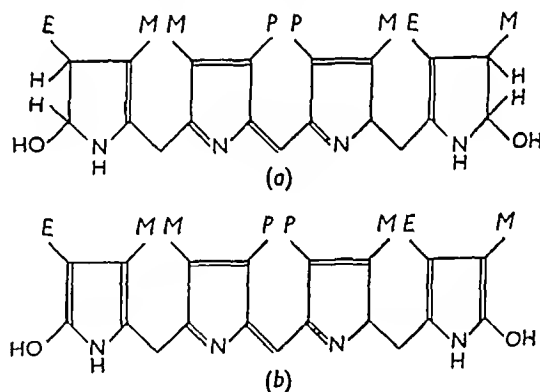


Fig 1 (a), tetrahydromesobilene-*b*, (b), mesobilene-*b*, *E*=ethyl, *M*=methyl, *P*=propionic acid.

Urobilins in faeces. Two related members of the urobilin class are generally obtained from both faeces and urine. Their structures are illustrated in Fig 1. In the present paper structure I (a) will be termed tetrahydromesobilene-*b*, and structure I (b), mesobilene-*b**. Repeated attempts were made to estimate the total amount of both these urobilins by reduction to their leuco compounds—the corresponding urobilinogens or mesobilanes—followed by

* This step represents a simplification of bile pigment nomenclature (cf Siedel, 1939). It is considered necessary because of the confused state of the nomenclature of the bile pigments and particularly of the urobilins, where tetrahydromesobilene-*b* may be called stercobilin or urobilin, and mesobilene-*b*, urobilin or urobilin IX α . The problem will be adequately discussed by Lemberg in a forthcoming monograph.

coupling with Ehrlich's reagent These attempts failed The pigments were, therefore, determined in the spectrophotometer as their hydrochlorides, this derivative being formed by acidifying the washings of the ether extract of the faeces (*vide supra*) with HCl The specific extinction coefficient $E_{1\text{cm}}^{0.1\%}$ at 492 m μ was found to be 147 by measurement of a crystalline sample of the hydrochloride of tetrahydro mesobilene *b* The extinction coefficient of mesobilene *b* was assumed to be equal to that of the tetrahydro compound

Mesobilene *b* While tetrahydromesobilene *b* is stable to FeCl₃ oxidation, mesobilene *b* is oxidized to a bilviolin This reaction, which was used in a semi quantitative fashion by Lemberg, Lockwood & Wyndham (1938), is carried out in the following way The aqueous solution of the urobilins remaining from the previous estimation is extracted with 10–15 ml of chloroform The extract is then evaporated to dryness on a water bath and the residue taken up in 5 ml of methanol A sample is suitably diluted with methanol, and its extinction coefficient measured at 492 m μ , thus giving a measure of the tetrahydromesobilene *b* plus mesobilene *b* To 4.5 ml of the remaining methanol solution are added 0.5 ml of 20% (w/v) FeCl₃ in HCl The solution is refluxed in a test tube for 15 min, cooled, the pigments taken into ether with sodium acetate and after thorough washing to remove as much unchanged tetrahydromesobilene *b* as possible, the violins are extracted with approx 2.8N HCl The acid extract is read in the spectrophotometer at 492 and 560 m μ The violin absorption is negligible at the former wave length, 492 m μ , the absorption here being that of traces of tetrahydromesobilene *b*, the absorption of the latter compound at 560 m μ is 0.07 times that at 492 m μ , this value is subtracted from that found at 560 m μ , thus enabling the true violin absorption at this wave length to be obtained Not enough of the violin was obtained to enable a determination of its extinction coefficient but Lemberg, Lockwood & Legge (1941) have found the specific extinction coefficient of a strongly basic violin to be the same as that of biliverdin, $E_{1\text{cm}}^{0.1\%} = 46$ This value was taken for the violin obtained from the mesobilene *b* A sample of mesobilene *b* prepared 5 years previously by Lemberg *et al* (1938) from crystalline mesobilene (mesobilinogen) gave a mixture of bilviolin and biliverdin on oxidation, and being analyzed by the above procedure gave 120% together of these two bile pigments calculated on the original mesobilene *b* The compound in faeces gave no biliverdin on oxidation This difference in behaviour prevented a more accurate check on the procedure

Abnormal blood pigments The blood was diluted, saturated with coal gas and examined with a hand spectro scope A band in the 620–630 m μ region of the spectrum could denote methaemoglobin, sulphaemoglobin or choleglobin A weakening or disappearance of the band on reduction with hyposulphite (Na₂S₂O₄) indicated the presence of methaemoglobin Alkali was now added, a weakening or disappearance of the band indicated the presence of sulphaemoglobin The remaining band is that of CO cholehaemochromogen (Lemberg, Legge & Lockwood, 1941)

RESULTS

The excretion of the porphyrins and urobilins was measured over periods up to 20 days in about 100 rats The data may best be summarized by reference

to the results obtained in an experiment in which 12 rats were fed on diet *B* (group *J*) They were kept in metabolism cages for 12 days before dosing was commenced with 1 g/kg of sulphanilamide daily Analyses were commenced 6 days before the dosing commenced and continued for a further 18 days The mean values for the 3-day excretion of urinary coproporphyrin, mixed faecal porphyrins, mixed

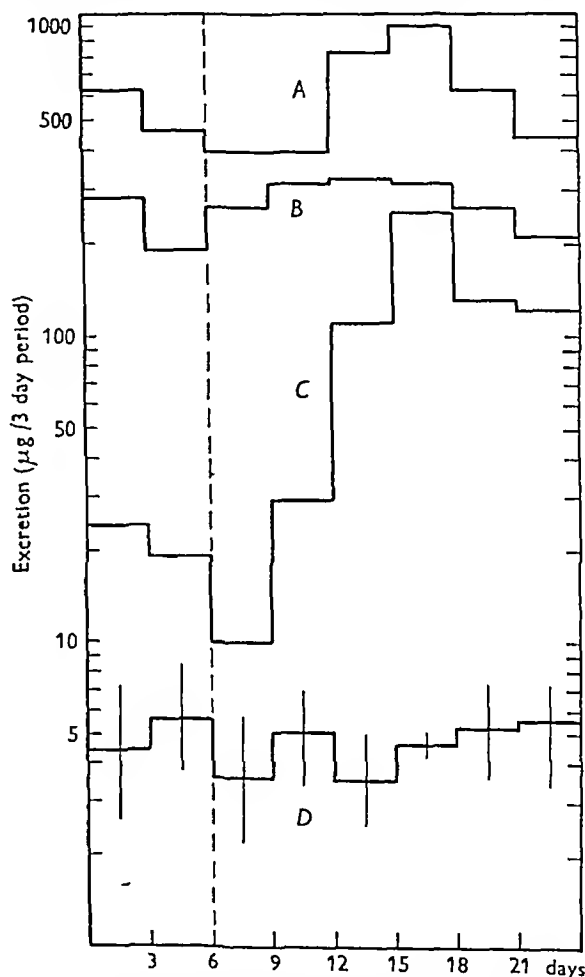


Fig 2 Mean excretion/3 day rat period of *A*, faecal urobilins, *B*, faecal porphyrins, *C*, faecal mesobilene *b*, *D*, urine coproporphyrin The vertical dotted line indicates commencement of dosage with sulphanilamide The vertical unbroken lines indicate range of values of urinary coproporphyrin

faecal urobilins and faecal mesobilene-*b* are plotted in Fig 2 on a logarithmic scale so that the quantitative relations between these substances may be more easily appreciated The range of values found for the excretion of the urinary coproporphyrin within the group at each period is indicated by the vertical lines on the appropriate graph in Fig 2 The range of values found for the excretion of each of the other derivatives is given separately in Table 1 The results from animals which did not survive a particular 3-day period were excluded from computation of the mean

Table 1 *Excretion of faecal pigments by group of rats dosed sulphanilamide (1 g/kg)*

Days	Control period		Period of sulphanilamide dosing					
	3	6	3	6	9	12	15	18
Porphyrins								
Range ($\mu\text{g}/3$ days)	170-420	140-260	170-400	160-370	130-430	120-570	140-330	160-310
Urobilins								
Range ($\mu\text{g}/3$ days)	430-910	320-660	190-610	190-560	410-1880	530-1890	360-1020	350-530
Mesobilene <i>b</i>								
Range ($\mu\text{g}/3$ days)	14-28	7-24	7-38	12-160	22-160	57-400	78-610	33-190
Surviving rats	12	12	12	9	9	8	8	7

Urinary coproporphyrin

Throughout the period of the experiment, the mean excretion of this derivative remained about constant. This result was typical of those obtained in the other experiments. The control excretion was the same as that found in rats on diet *A*. The individual records of all the rats show only two cases, both on diet *A*, which are not in accord with the above statement. Rat 69 excreted 3.7 and 6.4 μg coproporphyrin during two control periods and 2.4, 5.3, 13.6 and 17 μg coproporphyrin during four periods in which it was dosed with 1 g/kg sulphanilamide. The animal did not survive a fifth period of dosing and was the only one in which the increase in excretion reported by other workers was found. Another animal, number 71, showed an unusually high excretion throughout the experiment. The coproporphyrin excretion for the control periods was found to be 11.0 and 10.0 μg , and on five succeeding 3-day periods of dosage 7.0, 9.9, 12.5, 19.4 and 10.6 μg respectively. At this point the experiment was discontinued, the rat surviving. No significance is attached to the fact that both these animals were male or were found to be in the same group. Inspection of individual records failed to reveal any change in the excretion of the urinary coproporphyrin in the periods prior to the death of the rat, nor did scatter diagrams show any correlation between excretion of total faecal porphyrins and excretion of urinary coproporphyrin, or between the number of reticulocytes/cu mm and the excretion of urinary coproporphyrin.

Insufficient pooled urinary porphyrins were obtained at the end of the experiments to permit identification by confirmed methods of the proportion of types I and III present. Some preliminary experiments by the method of Watson & Schwartz (1940) (elution by 35% aqueous acetone and by 100% acetone of the methyl esters of coproporphyrins I and III respectively from Brockmann's alumina) indicated that over 90% of the porphyrin in the urine of both normal and dosed animals was type III.

Faecal porphyrins

In none of the experiments were the faecal porphyrins observed to increase in amount following dosage. The general tendency following dosage was, indeed, a decrease from 300-400 to 200-300 $\mu\text{g}/3$ day period in rats on diet *A*. Although this decrease was not observed in the rats on diet *B*, the variation in excretion between different rats, as evidenced by the data in Table 1, was typical of the other experiments. The porphyrins from successive 3 day periods from each rat were retained, and in several experiments were further purified so that the proportion of coproporphyrin in the mixture might be determined. This was found to vary, apparently at random, between 10 and 30% of the total porphyrin. Neither the type of diet nor the dosage with sulphanilamide had a predictable effect.

Total faecal urobilins

The increase in the mean excretion of urobilins from between 500 and 600 μg to between 700 and 1000 μg /period, after 9-12 days' dosage with sulphanilamide, was observed in all the experiments. The highest excretion observed, 1890 μg , represented a 2.5-fold increase, while between one third and one quarter of the animals showed a twofold increase on the highest pre dosage excretion. The values in Table 1 indicate the considerable variation in the response. Some animals, indeed, failed to show any significant increase in excretion even when their erythrocytes dropped to half their normal level in 15 days.

There was no correlation between the doubling of the pre dosage excretion of the urobilins and the survival or death of the animal. After the dosage with the drug had continued for 12-15 days some increase was observed in the concentration of the urobilins in the urine. This was not measured quantitatively.

Faecal mesobilene-b

After the experiments had been in progress for some time, it was noticed that the colour of the dilute sodium acetate solution of the urobilins

changed in hue from a rich yellow to a pinkish yellow. This was shown to be due to the presence of increased amounts of mesobilene-*b*. At first the amount of this pigment present was estimated qualitatively by the intensity of the band at 634m μ , seen after the addition of copper acetate to a methanol solution of the urobilins, the Cu⁺⁺ oxidizing the mesobilene *b* to a violin and forming its copper salt while leaving the tetrahydromesobilene *b* unchanged. In later experiments the quantitative procedure outlined above was used.

The increase in the mean excretion of mesobilene *b* seen in Fig. 2 was also seen in the other group of rats whose mesobilene *b* excretion was followed throughout dosage. As with the excretion of total urobilins, the response was observed to be variable. Of the thirteen animals who survived 15 days' dosage with the drug, only three failed to show less than a three-fold increase in mesobilene-*b*. The greater part of the increase in the excretion of the total urobilins is, however, due to the increase of tetrahydromesobilene-*b*. Thus, rat 89 showed an increase in total urobilin excretion of 1400 μ g between the sixth and ninth days of dosage, of which only 150 μ g is mesobilene-*b*.

The mesobilene-*b* excretion of individual rats appeared unrelated to their survival or death during the experiment. Examination of the pre-dosage levels of mesobilene *b* of rats on diets *A* and *B* indicated, however, that the excretion was significantly less on diet *B* (Student's *t* test, *P* less than 0.001).

In addition to the increased mesobilene-*b*, another abnormal pigment was observed. The porphyrins extracted from the washed ether extract of faeces obtained from animals, at the same time as the mesobilene-*b* excretion had increased, were observed to be bluer in colour than extracts obtained at an earlier period. This was shown to be due to the presence of a strongly basic biliviolin. Insufficient was obtained to show whether it was identical with that obtained by ferric chloride oxidation of mesobilene *b*, but this appears probable. Rimington & Hemmings (1938) also found blue pigments in the acid washings of the ether extract of faeces from rats dosed with sulphanilamide.

General observations

Haemoglobinuria was observed on 8/465 rat days during control periods and on 19/662 rat days during dosage. The difference is not significant and it is improbable, therefore, that the phenomenon is caused by the drug.

A green pigment was occasionally observed in voided urine (cf. Manwell & Whipple, 1929). This is probably due to the breakdown of oxyhaemoglobin to choleglobin since the characteristic band of CO cholehaemochromogen could be observed after appropriate treatment. In addition, flocks of bili-

verdin hydrochloride (showing the characteristic band at 670m μ) were obtained in the 0.14N-hydrochloric acid extract of the ether solution obtained in the course of routine analysis of the above urines for coproporphyrin.

When dosage with sulphanilamide was commenced, the increase in volume of urine observed by Rimington (1939) was not found (cf. Brownlee, 1939).

The amount of faeces generally showed an initial decrease when dosage commenced, rising after the first 2 or 3 days to the usual level.

There was generally a slight initial weight loss from which the animals recovered. Group J showed a drop in mean weight from 245 to 216 g in the 10 days spent in cages prior to commencement of dosing. During dosing the mean weight of the survivors fluctuated between 200 and 220 g.

In all the experiments the rats became cyanosed. The main abnormal pigment proved on examination to be sulphaemoglobin, with smaller amounts of methaemoglobin and choleglobin present. In this respect also the results differ from those of Rimington (1939), who only reported methaemoglobin to be present.

The survivors of the group on diet *B* showed an average drop in erythrocytes from 9.2 to 5.4 millions/cu.mm after 12 days' dosage, while the reticulocyte count rose at the same time from 1.8 to 7%. A similar result was found in another group.

The mortality observed in these experiments was greater than that observed by other workers. In the group on diet *B* only seven twelfths of the animals survived 18 days' dosage at 1 g/kg level. The mortality among the rats fed on diet *A* was greater. The only obvious evidence of dietary abnormality observed in the rats on either diet was an occasional discharge of porphyrin-containing fluid from the glands of Harder, the so-called 'bloody whiskers'.

DISCUSSION

In these experiments the sulphanilamide produced a severe anaemia in the rats. No increase was observed in the urinary excretion of coproporphyrin or in the level of the faecal porphyrins. The increased content of urobilins in the faeces strongly suggests that the accelerated haemoglobin breakdown proceeds by a normal route forming bile pigments and not porphyrins. Certain animals, although anaemic, did not show significant changes in the total amount of urobilins excreted. It is known, however, that, owing to their destruction in the gut, the excretion of urobilins is an unsatisfactory index of the amount of haemoglobin catabolized. If it is assumed that the life of the red cell in the rat is the same as that found in other mammalian species, about 120 days (dog, Hawkins & Whipple, 1938; man, Shemin & Rittenberg, 1945; Joep, 1946), the normal excretion of the

urobilins in rat faeces represents a recovery of about 20 % of the prosthetic group actually destroyed

The conclusion drawn from the association between the increased destruction of haemoglobin and the increased output of the urobilins might, perhaps, be queried, on the grounds that the pigments are found in increased amount because there is less destruction in the gut. There is, indeed, some evidence (*vide infra*) of altered bacterial activity in the gut, but none that this leads to a diminution of the overall destruction of the urobilins

In the light of the results reported in this paper, the hypothesis put forward by Rimington (1940), as to the origin of the increased urinary coproporphyrin, can no longer be supported. The results found in the present series of experiments are not the only ones suggesting that the problem is more complicated. While Wien (1938) found an increased excretion of coproporphyrin after sulphanilamide, he failed to find any increase after 15 days' dosage with 3 g/kg sulphapyridine, although Rimington (1940) found a fivefold increase in coproporphyrin excretion under similar conditions. Prof. Rimington has kindly informed me that he has heard from Dr M. Guggenheim, Hoffmann-La Roche Ltd, Basle, of another instance, where, under slightly different conditions, no increase in the excretion of coproporphyrin was noticed in rats after sulphanilamide treatment.

While it is possible (cf. Figgo, Strong, Strong & Shanbrom, 1942; Bittner & Watson, 1945) that genetic differences may lie at the basis of the discrepancy between these results and those of Wien, of Rimington and of Brownlee, it seems more likely that they are of nutritional origin. The fact that substances with negligible bacteriostatic properties, such as aspirin, phenacetin, phenazone (Brownlee, 1939) or aniline (Rimington & Hemmings, 1939), produce the porphyrinuria make it unlikely that the explanation is to be sought in the diminished bacterial synthesis of vitamins in the intestine. It is, therefore, probable that exogenous factors are involved. Mortality under dosage is perhaps the most important biological criterion of the physiological condition of the animals. Since the mortality in the present experiments was greater than that reported by other workers it seems most likely that factors present in other diets were absent in that used in the present series. On this view the porphyrinuria may be simply part of the normal erythropoietic response, perhaps slightly deranged by the drugs, but not of any great pathological importance for the animals. It should be noted, however, that in the present experiments a reticulocyte response was certainly present.

On an alternative view, the diet on which a porphyrinuria is found after drug treatment is not as complete as one on which no such response occurs.

This seems less probable than the former hypothesis, but cannot at present be entirely excluded. Ellinger, Edgar & Lucas (1935) have reported a porphyrinuria in rats on certain diets, while the various deficiencies producing porphyrin staining of the fur and whiskers (cf. Daft & Sebro, 1945), have yet to be thoroughly investigated from the point of view of porphyrin metabolism. Raoul & Marnay (1945a, b) failed to find an increase in urinary coproporphyrin when rats on a diet deficient in pyridoxine or pantothenic acid showed a cutaneous excretion of proto porphyrin.

Finally, comment must be made on the increased excretion of mesobilene-*b*. The only previous observation which related to the amounts of mesobilene *b* and tetrahydromesobilene *b* excreted were those of Lemberg *et al.* (1938). They examined urines from patients with various complaints, but observed no association between increased excretion of urobilins in the urine and the proportion of mesobilene *b* to tetrahydromesobilene-*b*. Nor was there a significant association between increased mesobilene *b* excretion and liver damage. The increase in mesobilene *b* in the present series of investigations provides, therefore, a further approach to the complex physiology of the urobilins. Baumgartel (1943a-d), in a series of papers available to the author only in abstract, has reported experiments which indicate that bacteria cannot reduce biliverdin, and that only after this compound is reduced to bilirubin by the liver (Lemberg & Wyndham, 1936) does bacterial catalysis of further hydrogen transfer commence. The complete reduction of the conjugated double bond system of the bilirubin is reported by Baumgartel to follow a series of steps in which (1) the α β double bonds of the terminal pyrroles are reduced, (2) the hydrogens shift from this position to the α and c methene bridges, forming mesobilane, the precursor of mesobilene *b*, (3) the α β double bonds of the terminal pyrroles are again reduced forming tetrahydromesobilane, the precursor of tetrahydromesobilene *b*. On this hypothesis the increased amounts of mesobilene *b* found after dosage with sulphanilamide result from a partial inhibition of step (3). It is uncertain whether such an inhibition takes place at the enzyme level, or whether it is due to alterations in the composition of the bacterial population. It would be useful to reinvestigate the varieties of bile pigments excreted following the administration of other sulpha drugs (cf. Greenblatt & Greenblatt, 1945).

SUMMARY

1. After treatment with sulphanilamide, rats were observed to show increased excretion of urobilins. The reported increase in urinary excretion of coproporphyrin after such treatments was, however, not found.

2 It is concluded that such a porphyrinuria does not derive from an abnormal breakdown of the prosthetic group of haemoglobin, but in some other way. Dietary deficiencies are thought to be responsible for the failure to observe this phenomenon in the present experiments.

3 The increase in faecal excretion of the urobilins was not noticed in all the rats treated with the drug, but in most there was a marked increase in the ratio

of mesobilene *b* to tetrahydromesobilene *b*. The latter phenomenon is probably due to the influence of the drug on the flora of the intestines.

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Acid-soluble Pigments of Shells

1 THE DISTRIBUTION OF PORPHYRIN FLUORESCENCE IN MOLLUSCAN SHELLS

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Red fluorescence in marine shells was described during the last century by McMunn (1886), and later by Furrer & Querner (1929), who drew attention to the importance of fluoroscopy as an aid to systematics. In 1930 the first of a series of observations by Fischer and his co-workers (Fischer & Jordan, 1930, 1934, Fischer & Hoffmann, 1937) dealt with the isolation from shells described as *Pteria radiata*, one of the group of pearl mussels, of a porphyrin intermediate in character between uroporphyrin and coproporphyrin. This substance was named conchoporphyrin. Uroporphyrins were isolated from other species of *Pteria* (Fischer & Haarer, 1931, Waldenstrom, 1937, Tixier, 1945), and from a species of *Glanculus* and several Trochidae, whose fluorescence

had been observed by Querner (Tixier, 1945). Fischer had noticed the absence of red fluorescence in *Helix pomatia*, but since Tixier's paper no serious attempt seems to have been made to determine the exact systematic range of shell porphyrins among the mollusca. A study by Turek (1933) details a wide range of fluorescent colours, some at least of which do not appear to be true examples of fluorescence, but the only other study of molluscan porphyrins in recent years is that of Dhéré & Baumeler (1928) on the dermal porphyrin detected by McMunn (1886) in *Arion*.

In the present investigation an attempt was made to ascertain by fluoroscopy the extent of the tendency throughout the mollusca to deposit shell por-

phyrin. A serious criticism of the work of Fischer arises from the inadequacy of his identification of the species under study—much of his investigation was based on polished and unlocalized material. In the case of *Pteria radiata* in particular, a member of a genus which has been heavily overdescribed, identification of cleaned shells is almost impossible. The differences between the porphyrins isolated from this species and from related forms render a higher degree of systematic accuracy essential. We do not claim to have overcome this standing difficulty. The present survey covers the whole field of molluscan shells, and the help given us by experts in the various groups does not wholly compensate for a lack of detailed identification. As an arbitrary measure of reliability, throughout this communication specific names marked * are based upon the tablet names in the Natural History Museum; unmarked names are derived from material in private collections, for the identification of which we must accept responsibility.

EXPERIMENTAL

Criteria of porphyrin deposition

The specificity of red fluorescence in molluscan shells, on which we relied as a primary guide to the presence of porphyrin, rests on fairly firm ground. Reddish fluorescence of non-porphyrin origin has been mentioned in connexion with only four molluscan pigments, in *Halotis californiensis* (Lemberg, 1931), in a phase of 'halotirubin' (Lederer, 1940) which may be the same material, in the blue phase of chromodorrin, which shows 'slight reddish fluorescence' (Crozier, 1916), and in aplysiopurpurin (Derrien & Turemi, 1925). Pink and orange fluorescence found by us in several species is described below.

Porphyrins contained in shell are at least as stable as the visible shell pigments, and old museum material has the advantage of being free from non-specific emission due to muscle and fixatives. Much of the material in the Natural History Museum is upwards of 100 years old, but intense red fluorescence was obtained in many instances, and its brightness was fully equal to that found in fresh material of the same species. Marked porphyrin fluorescence was found in specimens of *Gibbula cineraria* from the Clyde beds (post-Pleistocene), in *Pteria medea* from the London Clay, and in several species from the Calcaire Grossier (Paris basin, upper Eocene)—*Fissurella squamosa* Desh., *Angaria calcar* Lk., *A. lima* Lk., and *Tectus crenularis* Lk. In these four species no visible pigment whatever remained.

Methods

Shells of about 3000 species, including pelecypods, land and fresh water gastropods and a few scaphopods, were examined under an 80 W Osira mercury arc mounted in a

Wood's glass envelope. The amount of visible light passing this filter was insufficient to obscure the fluorescence in any but the most highly polished forms. Following the experiment of Fischer in direct spectroscopy of the shell, we also examined a number of species, both those found to be fluorescent and others showing non-fluorescent pigments of various kinds, by this method. A 100 W opal lamp was mounted in a box about 3 cm. below a black metal screen in which a small hole had been made. This screen carried a glass sheet to protect the specimen from heat, and over the hole was mounted a Hartridge reversion spectroscope (Beek). In the case of very rough shells, it was occasionally found necessary to mount the specimen on the arm of a 50 cycle buzzer, in order to secure a uniform field. The presence of porphyrin bands was taken as confirmatory evidence.

Where porphyrins were found, and material for sacrifice was available, acid extracts of the shell were made and examined directly by means of the reversion spectroscope, and a Hilger quartz ultraviolet spectrograph, using a ribbon filament lamp as source. Details of the separation and character of the pigments found will appear in a separate paper.

RESULTS

Two main shades of porphyrin emission, a scarlet and a paler eosin-pink, were detected, the second being usually found in thin species, but both being present in *Aplustrum*. Beside these typical porphyrin shades, we noticed several other colours of fluorescence, usually of low intensity. Nacreous shell usually fluoresces blue, and adductor scars white. Yellow tints are often due to the presence of varnish, but true yellow emission occurs in some examples of *Proserpina nitida*, and in the pedal slime of *Helix aspersa* (Turemi, 1926). Several species of *Cypraea*, especially the orange-brown variant of *C. tigris*, show orange emission, and we found a similar colour in the lip of *Papuna bovini*, in the mark corresponding to the visceral hump of *Patella depressa*, in *Alcadia* and *Tellina* spp. and elsewhere. The eggs of *Subulina* have an intense white fluorescence, and can be seen through the parent's shell.

Distribution of porphyrins in marine genera

Porphyrin deposition in marine molluscs centres around the following genera, in which it is wide spread.

Gastropoda	Pelecypoda
Fissurellidae	<i>Placuna</i> , <i>Enigmonia</i>
Trochidae (many genera)	<i>Pinna</i>
<i>Angaria</i>	<i>Pteria</i> , <i>Pinctada</i>
<i>Leptothyra</i>	<i>Isognomon</i>
<i>Lithopoma</i>	<i>Vulsella</i>
<i>Tricoha</i>	<i>Malleus</i>
<i>Erato</i>	
<i>Trivia</i>	
<i>Hydatina</i>	
<i>Bulla</i>	
<i>Aplustrum</i>	
<i>Haminoea</i>	
<i>Umbraculum</i>	

In the following additional groups, the occurrence of shell porphyrin is sporadic. The number of species in which it was detected is added, a star (*) indicating that there are probably others

Gastropoda		Pelecypoda	
<i>Acmaea</i>	1*	<i>Anomia</i>	1
<i>Torinia</i>	1	<i>Clausmella</i>	1
<i>Cypraea</i>	6	<i>Gafrarium</i>	1
<i>Marginella</i>	3	<i>Sunetta</i>	1
<i>Velutina</i>	2*		
<i>Actaeon</i>	1*		

Beside these, a few species of *Theodoxus*, a freshwater genus closely related to the marine Neritidae, produce traces of shell porphyrin. Among gastropods, there is a focus of porphyrin deposition in the primitive groups of marine forms (Archaeogastropoda), with another among tectibranch opisthobranchs, and another in an isolated group including *Erato*, *Trivia*, *Velutina*, and a few *Cypraea*. Whereas of 100 odd species of *Cypraea* and fifty of *Marginella*,

only six and three species respectively were found to deposit porphyrin, almost all the species of *Trivia* examined (except *T. oniscus* and *T. nuxca*) are regularly fluorescent. The trochid group shades off into forms related to the Turbinidae, and it is already known, from work by Krukenberg (1883), Tixier (1945), and others, that linear tetrapyrroles (bilitrienes) play a part in the pigmentation of these shells. Perhaps at this point in molluscan phylogeny the power to open the porphyrin ring was developed. Porphyrins are frequently found in those gastropods whose shell is partially enveloped in life by the mantle lobes. In *Cypraea* particularly, the porphyrin seems to be associated with those parts of the pigmentary pattern which are laid down after the formation of the lip. In addition to the distribution in gastropods, which is set out in greater detail in Table 1, traces of pink fluorescence were noted in the scaphopods *Dentalium entalis*, *D. formosum*, and the Loricates *Ischnochiton herdmanni* and *Tonicia ceylonica*.

Table 1 *Porphyrin fluorescence distribution in marine species—gastropoda*

(S=Scarlet, E=Eosin pink)

	Emission tint	Conformity with pigment pattern	Site	Intensity
* <i>Clypidina notata</i>	S	Yes	Tip and interior	Medium
* <i>Fissurella peruviana</i>	S	Yes	Days and interior	Medium
<i>F. maxima</i>	S	Yes	Rays, bevel of lip	Strong
<i>F. pulchra</i>	S	Yes	Rays, bevel of lip	Medium
* <i>F. latemarginata</i>	S	Yes	General	Very strong
<i>Lucapina crenulata</i>	S	No	Diffuse	Medium
<i>Acmaea virginea</i>	E	Yes	Rays	Weak
* <i>Trochus niloticus</i>	S	Yes	Red areas, apex	Medium
* <i>T. sandwicensis</i>	S	?	Red areas, apex	Medium
* <i>T. pyramis</i>	S	No	Area on columellar lip	Strong
<i>Clanculus puniceus</i>	S	Yes	Red areas	Strong
<i>C. clangulus</i>	S	Yes	Purple areas	Medium
* <i>C. pharaonis</i>	S	Yes	Red areas	Medium
<i>C. floridus</i>	S	Yes	Red areas	Very weak
* <i>Ethalia guamensis</i>	S	Yes	Umbilicus	Strong
* <i>E. striolata</i>	S	Yes	Shell surface	Medium
* <i>E. zelandica</i>	?	—		
* <i>Isanda coronata</i>	S	Yes	Shell surface	Medium
* <i>I. pudibunda</i>	S	Yes	Shell surface	Medium
* <i>I. rhodomphala</i>	S	Yes	Umbilicus	Strong
* <i>Monilia calyculus</i>	S	No	Umbilicus and columellar lip	Medium
* <i>M. philippini</i>	S	Yes	Umbilicus	Medium
* <i>M. lifuana</i>	S	No	General	Trace
<i>Thalotia comica</i>	?	—		
<i>Elenchus bellulus</i>	?	—		
<i>E. irsodontes</i>	?	—		
<i>Umbonium vestiarum</i>	S	No	General	Very faint traces
* <i>U. giganteum</i>	S	Yes	Lip and umbilicus callus	Strong
<i>U. suturale</i>	S	Yes	Umbilicus callus	Strong
* <i>U. javanicum</i>	S	?	Bands, sutures, callus	Strong
* <i>U. conicum</i>	S	No	General	Medium
<i>U. costatum</i>	S	Partial	Lip and callus	Strong
<i>U. australe</i>	S	Yes	Bands, lip and callus	Strong
<i>U. moniliferum</i>	S	Yes	Callus only	Strong
<i>Livona pica</i>	S	Yes	Black areas	Strong
<i>Gibbula magus</i>	S	Yes	Red areas	Strong
<i>G. cineraria</i>	S	No	Apical callus	Weak
<i>Monodonta colubrinum</i>	S	—	General	Strong
<i>M. confusum</i>	S	—	Patch near denticle	Traces
				Weak

Table 1 (cont)

	Emission tint	Conformity with pigment pattern	Site	Intensity
* <i>Angaria</i> spp	S	No	General	Strong
<i>Leptothyra sanguinea</i>	S	Yes	Red areas	Medium
<i>Lithopoma americana</i>	S	No	Patch on columellar lip	Very strong
<i>Astraea triumphans</i>	S	Yes	Umbilicus callus	Strong
<i>Tricolia pullus</i>	S	Yes	Individuals	Medium
<i>T. clongata</i>	E	Yes	Yellow individuals	Weak
<i>Theodoxus fluviatilis</i>	S	Yes	Individuals	Traces
<i>T. prevostianus</i>	S	No	Patchy	Traces
<i>Neritodryas dubius</i>	S	Yes	Pink bands, red form only	Strong
* <i>Neritina communis</i>	S	Yes	Pink bands	Strong
<i>Torinia variegata</i>	S	Yes	Red areas	Medium
* <i>Velutina laevigata</i>	E	—	Orifice, young shells	Weak
<i>V. zonata</i>	E	—	—	Traces
<i>Erato vitellina</i>	S	Yes	General	Strong
<i>E. lacus</i>	S	No	Lip only	Strong
<i>E. lachryma</i>	S	Yes	Bands	Strong
<i>E. columbella</i>	S	Yes	General	Strong
<i>E. cimaculata</i>	S	Yes	Spots	Strong
<i>E. sulcifera</i>	S	Yes	General	Medium
* <i>Trinia ovulata</i>	S	No	General	Weak
<i>T. archica</i>	S	No	General	Strong
<i>T. monacha</i>	S	No	General	Strong
<i>T. quadripunctata</i>	S	No	General	Medium
<i>T. pediculus</i>	S	Yes	Not dark areas	Medium
<i>T. oryza</i>	S	—	—	Traces
* <i>T. merces</i>	S	Yes	Dark areas only	Medium
<i>Cypraea cinerea</i>	S	No	General, esp extremities of mouth	Strong
* <i>C. mappa</i>	S	No	General	Very strong
* <i>C. subviridis</i>	S	No	General	Weak
* <i>C. pulchra</i>	S	No	General	Weak
* <i>C. isabella</i>	E	—	—	Trace
<i>Marginella ornata</i>	S	No	General in young shells, later red areas	Medium
* <i>Hydatina physis</i>	E	No	Transverse growth lines	Weak
<i>Bulla ampulla</i>	S	Yes	Pink and purple areas	Medium
<i>B. adansonii</i>	S	Yes	Pink and purple areas	Medium
* <i>B. quoyi</i>	S	Yes	Pink and purple areas	Medium
<i>Actaeon tornatilis</i>	S	Yes	Pink bands	Medium
<i>Haminea vesicula</i>	E	Yes	General	Strong
* <i>Aplustrum amplustre</i>	E, S	Yes	Lilac and pink bands	Strong
<i>Umbraculum mediterraneum</i>	S	Yes	Visceral stain	Traces

* Identification based on British Museum labels

Table 2 Other colours of emission in Gastropods

	Emission tint	Site
<i>Proserpina nitida</i>	Yellow	General
<i>Helix aspersa</i>	Yellow	Areas contaminated with foot mucus
* <i>Alcadia rhodostoma</i>	Yellow	Lip and operculum
<i>Cypraea tigris</i>	Orange	Shell of orange form
<i>Patella depressa</i>	Orange	Visceral stain
<i>Murex regius</i>	Orange pink	Lip
<i>Fasciolaria tulipa</i>	Orange pink	Lip
<i>Papuna boivini</i>	Orange	Lip
<i>Subulina octona</i>	White	Contained eggs

* Identification based on British Museum labels

Land and fresh water gastropods

With the exception of *Theodoxus*, we detected no porphyrin in the shells of any fresh water forms. Long series of land operculates and pulmonates of all groups were equally negative. The observation of

porphyrin in the dermis of slugs, while telling against any argument based on photosensitizing effects, has no counterpart in our study of shells.

Pink or orange-pink fluorescence of a very low order, not extractable by acid, was observed in a number of land shells. The species concerned were

all white, xerophilic or desert forms, and mostly in a weathered or sunbaked state, a control series of fresh material being negative. The pigmentation was patchy, confined in some cases to the brown bands, and in others to areas which had been in contact with the ground or with moisture. The species concerned were *Rumina decollata*, *Ena detrita*, *E. exilis*, *Strophia uva*, *Geomitra nitidiuscula*, *Otala lactea*, *Eremina desertorum*.

One species (*Bulinulus reebsi*) showed intenser orange pink fluorescence, not typical of porphyrin, and most marked in the youngest parts of the shell. No material was available for extraction.

We regard this pink fluorescence as of non-porphyrin origin, due possibly to the existence of fluorescent, crystalline forms of calcium carbonate in old shells.

Pelecypoda

Beside *Pteria* (*Pinctada*), whose fluorescence was first noted by Fischer & Jordan (1930), porphyrins occur in some Anomidae (*Placuna* and *Enigmonia*), in *Pinna*, and in *Malleus*, *Isognomon*, *Vulsella*. The only higher bivalves in which we found any trace of red fluorescence were *Venus* (*Clausinella*) *fasciata*, *Gafrarium divaricatum* and *Sunetta solandrei*. Apart from these three species, the porphyrin-containing bivalves have a superficially similar shell structure and a tendency to produce rays of brownish or purplish pigment which, in many individuals, is non-fluorescent. *Malleus vulgaris*, which produces large amounts of easily extractable acid-soluble violet pigment, shows streaks of porphyrin in the region of the hinge. The extract is not fluorescent in solution. *M. regula* was found to contain small amounts of extractable porphyrin with large quantities of other, chromatographically distinct, pigment.

Anatomical distribution

Shell porphyrin fluorescence is of four main types: (i) Generally diffused, (ii) sharply coincident with the visible pigmentary pattern, (iii) localized in non-pigmented or faintly pigmented areas which are constant for the species, (iv) confined to one or more bands in a visible pigmentary pattern.

Forms showing general diffusion include some *Trivia*, and several *Fissurella*. Pigmentation of the second type is of great interest, since porphyrin may be absent from the pigmented areas of two otherwise identical shells, and its intensity bears no relation to that of visible pigment. The third type of distribution suggests the association of porphyrin deposition with a particular organ, the visceral hump of *Umbraculum*, or the columellar mantle in some *Trochi*. Univalves frequently show localization, if any, in the region of the mouth, porphyrin being confined to the umbilical callus in some *Umbonium*,

to a pink band in others to a patch on the lip in *Lithopoma*, to the callus occupying the site of the old protoconch in *Gibbula cineraria*, and, as already mentioned, to the chlamydogenous areas of many Cypræids. Coincidence with a band, usually pink, is most marked in *Aplustrum* and some species of *Umbonium* and *Neritina*. In *Pinna*, pigment is often confined to a small area inside the valve and near the middle.

Coincident pigment The visible pigments of porphyrin-containing forms show an emphatic preponderance of purple, red, and brown. In pigmentary patterns of type (ii) above, the coincident pigment may be black (*Livona pica*), scarlet (*Trochus niloticus*, *Clanculus puniceus*), pink (*Aplustrum amplustre*) or brown (*Pteria*).

In some cases there is definite correlation between the brightness of fluorescence and the depth of colour of the seasonal pigment varices, but closely related species, or individuals of the same species, of identical colour, show wide variation in the porphyrin content, and in almost all instances the porphyrin fraction is chromatographically separable from the main coloured material. In *Trivia pediculus* the whole dorsum except the brown spots fluoresces in the closely similar *T. merces* the position is reversed.

Site In no instance have we found porphyrin in nacreous shell, though it may occur in callus laid down by the somatic parts of the mantle. In many forms it is sharply confined to the surface layer of the shell, next to the periostracum, where this is present. In *Placuna*, which has a lamellar structure rather like mica, the pigment is sandwiched between the layers of shell.

Relation to haemoglobins

Molluscan haemoglobins may be circulatory or confined to the radular muscle. We could not establish any relation between the occurrence of pyrrolic respiratory pigments and the presence of shell porphyrin. Forms such as *Arca* and *Planorbis*, which are known to have red blood, are devoid of shell porphyrin.

Individual variation

Of twenty specimens of *Venus* (*Clausinella*) *fasciata*, only five showed fluorescence, and in two of these intense fluorescence was noted. One of the brightest specimens belonged to the permanganate-coloured variant, though comparable purple variants of *Dosinia* and several other Veneridae contained no detectable porphyrin. Fluorescence in the other two venerids was likewise confined to pink or purplish specimens.

In *Trivia monacha*, *Cypraea isabella*, *C. mappa*, and *C. cinerea*, non-fluorescent individuals are not

uncommon, and intermediate forms showing porphyrin only round the mouth and in the terminal calli also occur

Fluorescence is often most marked in small examples of a species, as though dilution by shell matter were taking place

Individual variation in most forms is as marked as variation in the visible intensity of pigmentation, and it is, therefore, impossible to exclude porphyrin formation without examining long series. In the light of this finding, a number of our negative results may well be revised by more extensive studies

Relation to body colour

Since shell porphyrins are almost certainly a product of gradual accumulation, high concentrations of fluorescent material are not necessarily to be expected in the mantle. This important study could not be pursued in the most suitable forms, such as *Pinctada*, but in the British *Trivia* fluoroscopy of the living animal showed no evidence of large tissue concentrations of porphyrins. The siphon and mantle borders exhibit slight reddish fluorescence, but the main pigment, an orange, acetone soluble material, proved to be a complex mixture of carotenoids, giving seven zones on chromatography, while extracts of the ground animal in 0.2N-hydrochloric acid showed no red fluorescence whatever if free from shell fragments. The bright orange faeces of *Trivia* are also without fluorescence

Relation to calcium metabolism

The irregularity with which porphyrins occur in shells, and the high concentrations which exist in many thin, poorly calcified forms, do not suggest that they play any major part in shell deposition, al-

though they are known to appear in the dart sac of *Helicids* during the process of calcification (Kuhnelt, personal communication). The extremely high concentrations present in many forms favour the idea that porphyrins are secreted with the shell as a means of disposal

Relation to molluscan taxonomy

Our findings for distribution agree well with the anatomical classification of Thiele (1929, 1931). Porphyrins, although absent from recent species of *Pleurotomaria*, and replaced by other pigments in *Halotis*, appear to be widespread among the less specialized Archaeogastropoda. Apart from outlying forms such as *Marginella* and the few Veneridae, the other porphyrin-depositing groups are almost equally clearcut. In *Cypraea*, for instance, the subgenus *Luria*, created on anatomical grounds, is a self-contained focus of porphyrin deposition. Even the superficial similarity of appearance between *Erato* and *Marginella*, unrelated forms, appears to be accompanied by a convergence of pigment metabolism. The comparative biochemistry of such resemblances should certainly be studied further

Direct spectroscopy of shells

Porphyrin spectra were obtained in a number of bivalves and in *Umbrella* by direct transillumination of the shell. Fischer & Jordan (1930) had examined '*Pteria radiata*' by this method, and found bands at

	6321-6168	5882-5650	5508-5408	5172-4979 Å
max	6244	5766	5458	5075

Our own readings for porphyrin-containing and control species were as follows (Table 3)

Table 3 *Absorption spectra of shells by direct transillumination*

	Abs maxima (A.)				Fluorescence
	Nil				Nil
<i>Umbraculum mediterraneum</i> (edge)	6220	5900	5465	5120	Yes
<i>U mediterraneum</i> (visceral stain)	6210	5804	5480	5010	Yes
* <i>Pteria chinensis</i>	6200	5955	5420	5095	Yes
* <i>P castanea</i>	—	—	—	5010	Yes
* <i>P rufa</i>	—	—	—	5015	Yes
* <i>P hirundo</i>	—	—	—	5035	Yes
* <i>P electrina</i>	—	—	—	5000	Yes
* <i>Enigmonia enigmatica</i>	—	—	—	5000	Yes
* <i>Placuna sella</i>	—	—	—	5070	Yes
<i>Venus fasciata</i> (pink)	—	—	—	5010	Nil
* <i>Umbraculum cumingi</i> (edge)	—	—	—	5010	Yes
<i>U cumingi</i> (visceral stain)	—	5975	5490	5000	Nil
<i>Chlamys tineta</i> (yellow form)	6900†	—	—	4950	Nil
<i>C tineta</i> (orange red form)	7100†	—	5140	5000	Nil
<i>C tineta</i> (red form)	6900†	—	5045	5000	Nil
<i>C tineta</i> (orange form)	7000†	—	5042	5000	Nil

Probable error in estimating maxima of very low intensity ± 20 Å

* Identification based on British Museum labels

† Limit of transmission in the red

The scope of the present work is limited to the general distribution of red fluorescence in shells. Of the spectral bands detected some at least are due to concomitant pigments of non-porphyrin character which have been separated chromatographically. Details of these, and of the chemistry of isolated shell porphyrins, will be published in forthcoming communications.

SUMMARY

1 The distribution of red fluorescence in molluscan shells is described. It is absent from land and fresh-water forms, and commonest in the Archaeogastropoda, appearing also in the Lamellariacea, certain Cypraeidae, the tectibranch opisthobranchs,

Umbraculum, the pearl oysters, and sporadically elsewhere. The limits of distribution coincide closely with the existing anatomical nomenclature.

2 Porphyrins in shells are commonly associated with a wide variety of other acid-soluble pigments.

3 Both these and the porphyrins are at present under fuller investigation, of which details will appear separately.

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Characterization of Sugar Components of Proteins

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Carbohydrates form an integral part of many biologically active complexes and the problem of their estimation in the presence of protein is common. The classical methods are inapplicable to the small amounts generally involved, and numerous colour reagents have been used for the detection of various sugars. Their number and the modifications of the few reactions which have been developed on quantitative lines reflect the existing state of dissatisfaction. A critical investigation of the problem seemed desirable.

The ideal of a specific visible absorption assay for each sugar has not been realized, nor has it been possible to find a reagent giving equal colour intensities for equal quantities of sugar, irrespective of the configuration, so that current methods necessitate qualitative characterization before quantitative estimations can be attempted.

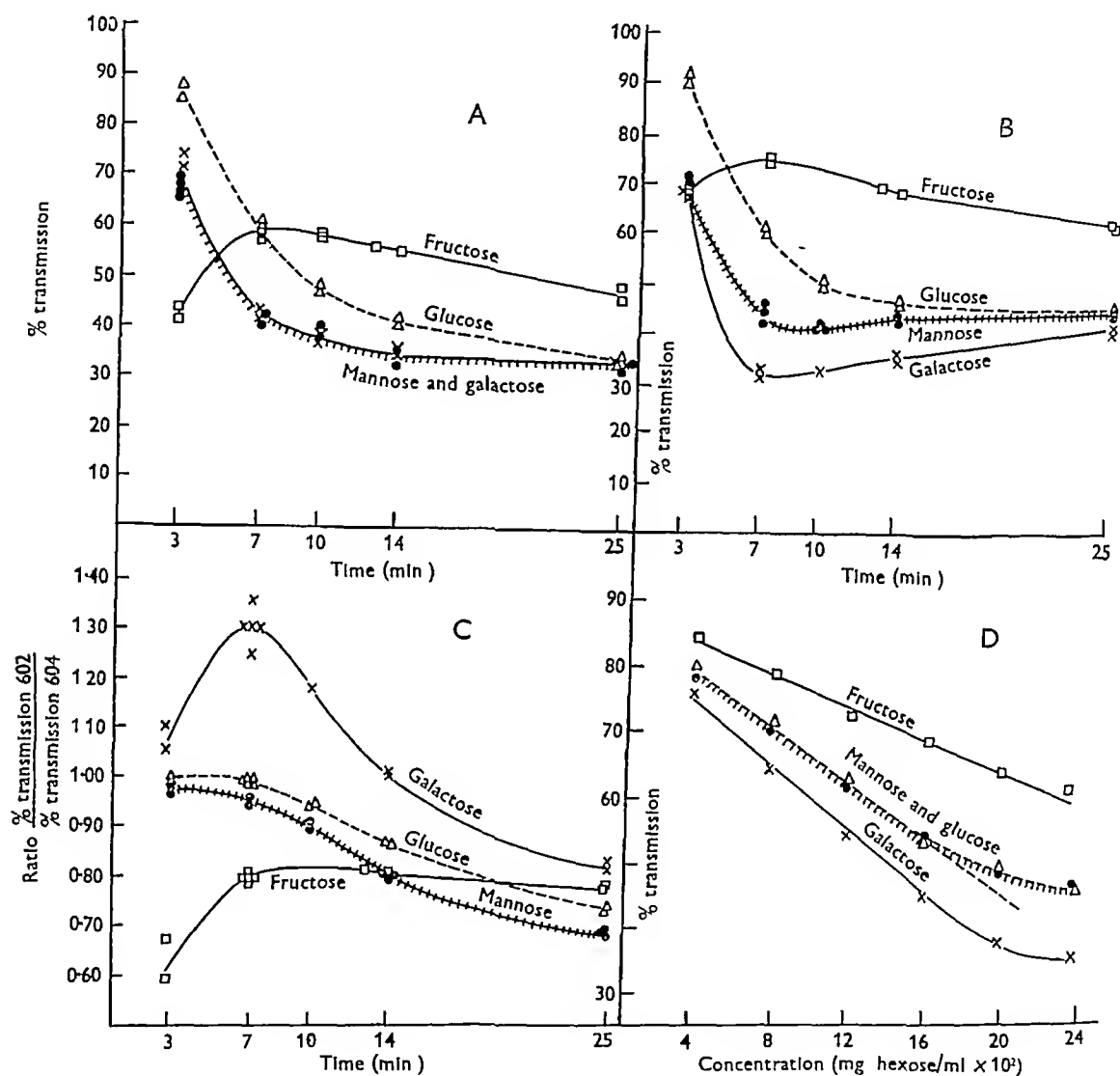
Colour reagents for sugars are either phenols or polyphenols with the substituents in the meta position (thymol, phloroglucinol, α - and β -naphthol, resorcinol, orcinol), or nitrogenous compounds, such as indole, skatole, diphenylamine, urea or guanidine. A range of thirty-two compounds of these two types was examined under standard conditions in concentrated sulphuric acid solution for colour formation with four representative hexoses, namely mannose, galactose, glucose and fructose. In every case colours were obtained, and distinction between mannose and galactose on the one hand, and glucose and fructose on the other hand, was possible. Mannose and galactose, however, showed similar absorption spectra. Fructose gave colour reactions more intensely and more quickly than the aldo sugars. The absorptions, especially in the case of the aldo sugars, were not intense enough to warrant a quantitative investi-

gation, particularly as many colours were not stable and sometimes the reagent itself gave a colour reaction in the absence of the sugar although the spectra of these 'blank' colours were usually distinct from those of the sugars

EXPERIMENTAL

Orcinol method

The procedure was essentially that of Sørensen & Haugaard (1933). Orcinol, usually pink as supplied, was recrystallized



Orcinol method the qualitative (A, B and C) and quantitative (D) determination of mannose +++●+++, galactose —x—, glucose --△-- and fructose —□— A, variation in percentage transmission with time (Ilford spectrum filter 602), B, variation in percentage transmission with time (Ilford spectrum filter 604), C, variation of the ratio $\frac{\text{percentage transmission with Ilford spectrum filter 602}}{\text{percentage transmission with Ilford spectrum filter 604}}$ with time, D, variation in percentage transmission with the concentration of the sugars (Ilford spectrum filter 604)

A closer investigation of the more promising reactions led to the conviction that of the two reactions which have so far been developed on quantitative lines, those with orcinol and carbazole are outstanding, with skatole as a possible third. A detailed comparison was, therefore, restricted to these three methods

from benzene until white crystals were obtained. A 1.6% solution (modification by Hewitt, 1937) keeps well when prepared precisely as described by Sørensen & Haugaard (1933), i.e. 1.6 g of orcinol is dissolved in 50 ml of distilled water, and the volume made up to 100 ml with the grade of H_2SO_4 specified for the reaction. Filtration through a sintered glass crucible over a thin layer of washed (30% H_2SO_4) supercel removed minute pinkish brown floccules

which appear to act as centres of decomposition and crystallization. Spontaneous crystallization may take place at temperatures below 16° and lower temperatures should be avoided. The reagent is light-sensitive and must be stored in the dark, it should be colourless and must be rejected when it acquires a pinkish-brown tint. In place of Sørensen's 2.0 ml, 2.5 ml of the reagent were used.

As qualitative characterization in this method is based on the varying rates of colour formation, necessitating the determination of colour development before completion of the colour forming reaction, rigid standardization is all important. Results obtained with reaction times of 3–7 min, which contribute most of the characteristic part of the time curve for any particular sugar (see Fig. 1), are reproducible only under optimum conditions of timing and temperature control. We aimed at an accuracy of ± 2 sec, (see also Rimington, 1940), and a maximum temperature variation of $\pm 0.3^\circ$. Since the oreinol colours are light-sensitive (Sørensen & Haugaard, 1933, Hewitt, 1937, Rimington, 1940), particularly those obtained with reaction times of 3–7 min, reproducible time curves are obtainable only when the solutions are shielded from light, not only during cooling before the tubes are read, but also during the heating on the water bath and while readings are taken on the absorptiometer. The reaction bath, of circular shape (Sørensen & Haugaard, 1933) with the tubes arranged around the periphery, was totally enclosed, an efficient stirrer being introduced through a hole in the centre of a sliding lid. Evaporation was minimized by floating a film of liquid paraffin on the surface of the water, the level of which was kept constant. The reaction was terminated by immersion in an ice bath. After 20 min in the dark, the tubes were transferred to wooden boxes with well fitting sliding lids, and after a further 30 min the colours were read in a photoelectric absorptiometer of the slide-back voltmeter type of conventional design (Müller, Garman & Droz, 1943). Ilford spectrum filters 602 and 604 were used in place of S 53, and S 43 (Sørensen & Haugaard, 1933, Rimington 1940). When all these precautions were strictly adhered to, time curves for the qualitative characterization of sugars were reproducible from day to day.

The quantitative estimations are less sensitive to variations. The time curves, obtained by plotting percentage transmission against reaction time, become parallel to the abscissa after 15 min (Fig. 1). Slight inaccuracies in timing or temperature control are thus not as important as in the steep part of the curve, and for the range 0.04–0.16 mg hexose/ml, a straight line relationship holds good, a convenient slope is obtained with Ilford spectrum filter 604 after 20 min heating. In the dark, the colours remained stable for several hours. It is not necessary to repeat standard curves every day. A weekly check, repeated when reagents were renewed, was found to be sufficient.

We can confirm the observations of Sørensen (1936) and of Hewitt (1937) that the slightly coloured solutions obtained by heating the protein solutions with the reaction H_2SO_4 in the absence of oreinol, do not represent true 'blanks'. They are a source of error when used as blank corrections in the conventional sense, and should be omitted.

The influence of amino acids on the reactions with the pure sugar solutions was tested by means of an amino acid mixture of the following composition: glycine 30, alanine 40, glutamine 40, histidine 2, arginine 2, cystine 4, trypto-

phan and tyrosine 0.5 parts. From the percentage transmission figures given in Table 1, it can be seen that there is some slight interference which increases with increasing amounts of amino acids. However, these slight deviations from the figures obtained with pure sugar solutions cancel out when filter 'ratios' are used for the characterization.

Table 1. *Orcinol method: effect of an amino acid mixture on percentage transmission for four hexoses*

(Concentration of the sugar solutions, 0.02%, time of heating, 7 min, i.e. time of largest contrast in colour response. Ilford spectrum filters 602 and 604. The figures are in each case the means of ten estimations.)

Sugar amino acid ratio	Percentage trans mission with filters		Ratio 602/604
	602	604	
Mannose			
Amino acid mixture	43.5	45.0	0.97
1 1	43.2	45.0	0.96
1 4	42.8	43.8	0.98
1 40	45.0	46.0	0.98
Galactose			
Amino acid mixture	43.2	32.2	1.34
1 1	44.2	33.5	1.32
1 4	44.0	33.2	1.32
1 40	44.8	35.0	1.28
Glucose			
Amino acid mixture	61.2	61.8	0.99
1 1	59.2	60.5	0.98
1 4	61.0	61.2	1.00
1 40	61.2	61.3	1.00
Fructose			
Amino acid mixture	59.0	73.2	0.81
1 1	58.2	72.8	0.80
1 4	59.2	74.0	0.80
1 40	60.2	74.8	0.81

Carbazole method

Gunn & Hood's method (1939) was strictly adhered to. The degree of purity of the reaction acid and the carbazole reagent are critical for success. Occasionally, batches of H_2SO_4 (A.R.) were unsuitable because of an impurity giving rise to fluorescence.

Purification of carbazole may be effected conveniently by chromatographic adsorption. Savory and Moore's alumina, standardized according to Brockmann, was used as the adsorbent and benzene as solvent. The impurities were retained on top of the column, even in the case of very impure commercial preparations. The narrow deeply coloured band on top may be separated into a deep blue, a yellow, a pink and a brown zone by subsequent washing with benzene. Washing was discontinued when the brown zone reached the bottom of the column and the carbazole was recovered from the eluate in 80% yield. One recrystallization from ethanol gave a white stable product. Less pure preparations become unsuitable for use within a few days. Even slight impurities caused the appearance of pink and green

tints in the blanks, making qualitative characterization impossible and introducing erratic errors in the quantitative estimations

The rate of colour formation was found to be approximately the same for all four hexoses, regardless as to whether the reaction was carried out at 60, 80 or 100°, except for fructose where maximum colour development is obtained after 5 min at 100°. Time curves are, therefore, not sufficiently distinctive for a qualitative characterization, but the differences in the specific absorptions between the various sugars are greater than in the orcinol method (Fig 2) and Gurin & Hood (1939) have made use of filter 'ratios'. The set of Ilford spectrum filter ratios used by us is indicated in Table 2

Table 2 *Carbazole method comparison of percentage transmission ratios obtained with mannose, galactose and glucose in the presence and absence of 40 times as much amino acids*

Ilford spectrum filter ratio	604 601	604 602	607 604	607 601
Mannose	1.76	1.09	1.54	2.78
Mannose + amino acids	1.75	1.11	1.50	2.78
Galactose	1.22	0.92	1.64	2.01
Galactose + amino acids	1.22	0.88	1.67	2.04
Glucose	0.71	0.64	2.02	1.44
Glucose + amino acids	0.74	0.66	2.02	1.51

Amino acids containing an aromatic nucleus interfere with the reaction (Gurin & Hood, 1939), and hexose figures obtained with the carbazole method are for this reason slightly higher than those obtained with the orcinol method (see Table 4). Table 2 shows that amino acids interfere little with the qualitative characterization. If the indicated range of ratios is used, differences due to amino acids cancel out. The same amino acid mixture as in the orcinol method was used.

Skatole method

Colour reactions of sugars with skatole were first described by Weehuizen (1907) who used hydrochloric acid. Dische & Popper (1926) investigated the reaction, using sulphuric acid. They did not, however, extend their investigations because skatole itself was found to give a red colour in the absence of sugar. We found that a deep purple was formed within 4 min on a boiling water bath and that the same colour is eventually reached on prolonged standing at a lower temperature. Although this colour is quite distinct from the 'sugar' colours (Fig 2), it is fairly intense. A specific absorption filter could not be devised, but it proved possible to reduce this blank value considerably by working at 80° instead of at 100° and choosing suitable concentrations of the acid and the reagent. Fig 3 shows that the optimum concentrations of sulphuric acid and skatole are 85 (v/v) and 0.5% (w/v) respectively, giving intense colour formation with the hexose and relatively little colour in the blank.

The skatole colours are light sensitive, and have to be protected as in the orcinol method. In the dark,

after heating times of 15 min, the colours are stable for several hours. Solutions which have been heated for 5–10 min darken more quickly, and should

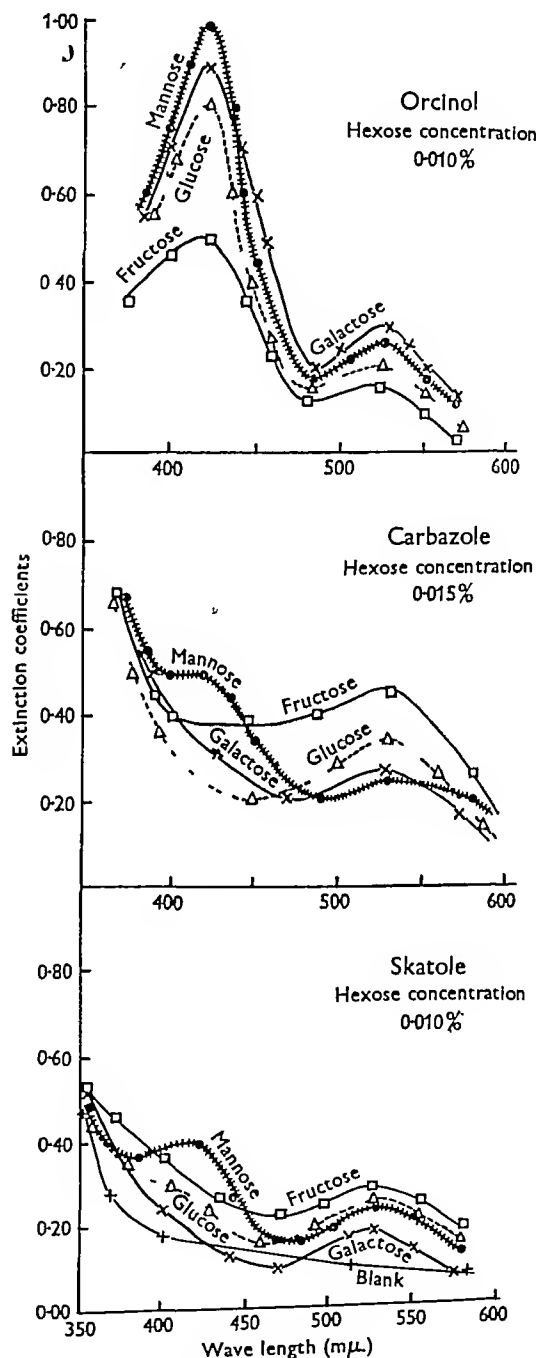


Fig 2 Photometric absorption curves for mannose —●—, galactose —x—, glucose —Δ— and fructose —□—, with the orcinol (top), carbazole (middle), and skatole (bottom) methods (Hilger medium quartz spectrograph)

always be read exactly 1 hr after removal from the water bath. Temperature control and timing is as critical as in the orcinol method and for the same reasons.

Apparatus The reactions were carried out in an enclosed water bath at $80 \pm 0.3^\circ$. Timing was carried out with a stop clock, and the colours were measured photoelectrically in 1 cm all glass cells.

Reaction acid 85% (v/v) H_2SO_4 (A.R.), obtained by adding 8.5 vol ice cold conc acid to 1.5 vol of water, with external ice cooling.

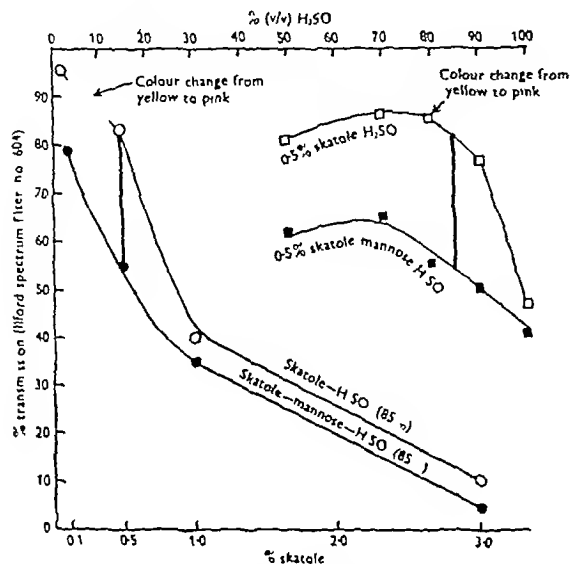


Fig 3 Top, effect of variation in sulphuric acid concentration on percentage transmission, —□— reaction of skatole with sulphuric acid in the absence of sugar, —■— reaction of mannose with skatole in sulphuric acid. Bottom, effect of variation in skatole concentration on percentage transmission, —○— reaction of skatole with sulphuric acid in the absence of sugar, —●— reaction of mannose with skatole in sulphuric acid (Ilford spectrum filter 604, time of heating at $80 \pm 0.3^\circ$ 15 min.)

Skatole Commercial preparations contain a yellow and a pink impurity. The former may be removed by recrystallization from light petroleum, the latter by recrystallization from aqueous ethanol until the base is obtained in a pure white state. A 0.5% (w/v) solution in 96% ethanol, which must be colourless, keeps practically indefinitely when stored in the cold.

Procedure 0.04–0.25 mg carbohydrate in 1 ml of solution was measured into wide boiling tubes (3.5 × 20 cm) and the solution cooled in an ice bath. 0.2 ml of the skatole reagent was added with a micropipette, followed by 9 ml of the reaction H_2SO_4 . Quick mixing was achieved by means of a flattened glass rod. The reaction was allowed to proceed at 80° in the dark and was interrupted at suitable time intervals by plunging in ice water. A standard time of 30 min in the dark was allowed before removing the tubes to the light protected boxes. Readings were taken after a further 30 min.

Qualitative characterization

1 **Specific absorption** A comparison with Fig 2 shows that the differences in the absorption spectra of four representative hexoses are greater than in the orcinol method and that the absorption curves are

similar to those obtained with carbazole. As in the latter method, qualitative characterization would be possible by means of a suitable set of percentage transmission 'ratios'.

2 **Rates of colour formation** As with orcinol, the rates of colour formation (Fig 4) vary with the configuration of the sugar, and allow better distinctions to be drawn between the sugars than the use of differences in specific absorptions. The light filters 601 and 604 were chosen for the construction of the time curves because their transmission ranges coincide roughly with the peak ranges in Fig 2.

3 **Use of ratios** By plotting a ratio of the percentages of light transmission obtained with the filters 601 and 604 against reaction time, characteristic curves were obtained for the various sugars which were independent of concentrations over the range where Beer's law is obeyed (Fig 4).

Quantitative estimations

Quantitative estimations are again less sensitive to experimental variation than qualitative characterization, for the same reason as in the orcinol method. Beer's law is obeyed within the range of 0.04–0.24 mg hexose/ml. Although the colours obtained after heating for 20 min were more stable, a period of 15 min was chosen because interference from the 'blank' colour becomes pronounced after this time. Filter 604 proved more suitable than 601.

Influence of amino-acids and glucosamine

In the presence of amino-acids, the initial mixing in the ice bath has to be done very gradually, or tints differing from those of the pure sugar solutions are obtained, and the shape of the time curves becomes distorted. If yellow tints are observed during the mixing in the ice bath, the particular tubes must be discarded. The amino acid mixture used in the previous experiments was again employed.

Fructose showed a greater initial rate of colour formation than the aldoses. An orange-red colour formed immediately on mixing in the ice bath, although the intensity reached in the water bath at 80° was not much greater than that obtained with the aldoses. The initial velocity of the reaction was such that the different stages could not be reproduced accurately during the gradual mixing of the reagents which is necessary in the presence of amino-acids, and fructose, although readily recognized, cannot be estimated in their presence. As in the other two methods, glucosamine, in quantities up to 40 times those of the hexoses, did not influence the reaction.

Application

When determining the hexose composition of an unknown mixture, it will first be necessary to remove as far as possible any admixed pigment. The sugar concentration must then be brought within the

working range of the given methods, i.e. 0.04–0.20 mg/ml. By comparing the tints, or better the absorption spectra, given by the unknown with the orcinol, carbazole and skatole reagents with those obtained with a number of different pure sugar

approximations in which all three methods play their part

A purified follicle stimulating fraction of pregnant mares' serum (P M S W III) assaying 250 i.u./mg protein was analyzed by all three methods. Fig. 5

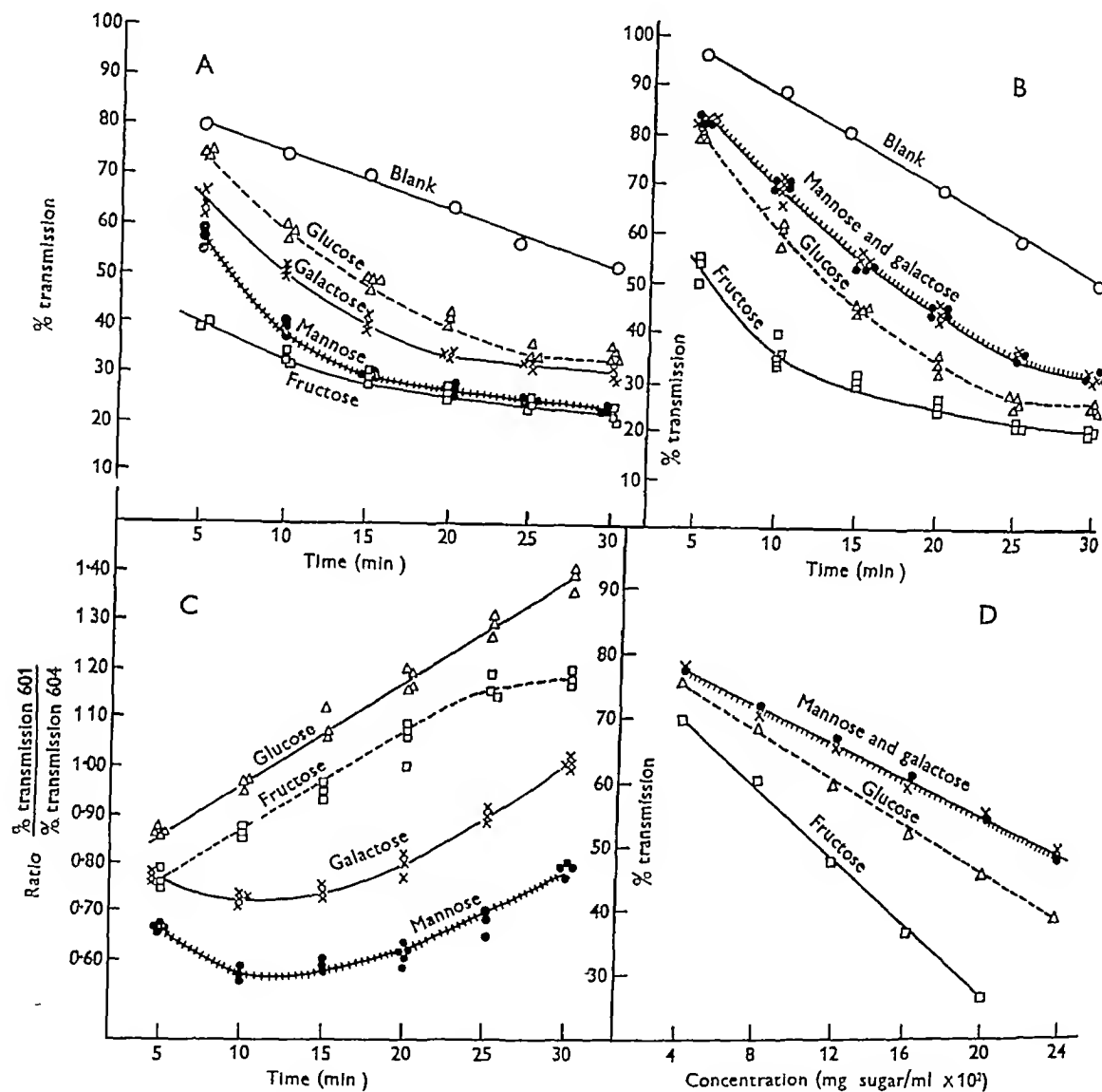


Fig. 4 Skatole method the qualitative (A, B and C) and quantitative (D) determination of mannose +++●+++, galactose —×—, glucose —△— and fructose —□— A, variation in percentage transmission with time (Ilford spectrum filter 601), B, variation in percentage transmission with time (Ilford spectrum filter 604), C, variation of the ratio percentage transmission with Ilford spectrum filter 601 to percentage transmission with Ilford spectrum filter 604 with time, D, variation in percentage transmission with the concentration of the sugars (Ilford spectrum filter 604)

solutions of similar concentration, a preliminary working hypothesis may be arrived at. The carbazole 'ratios' may then be used for a rough characterization which can be carried out quickly. The indicated possibilities can then be further investigated by means of time curves according to the orcinol and skatole methods. The approximate qualitative composition may thus be determined by a series of

gives a comparison of the ratio time curves of this sample with that of mannose, galactose and an equimolar mixture of these two sugars obtained with the orcinol and skatole methods. In both cases, the shape of the curve obtained with the P M S sample is consistent with the assumption that the carbohydrate component of this serum fraction is made up of equal amounts of mannose and galactose. The

results obtained with the carbazole method using four different percentage transmission ratios, in place of Gurin's (1942) one ratio, are also consistent with this view (Table 3). Fructose is certainly absent since no colour is formed with skatole in the cold. Although it is conceivable that the presence of glucose might

The carbazole and skatole values for hexose tend to be higher than those obtained with orcinol, but analysis shows that these differences are statistically insignificant as far as the skatole and orcinol methods ($P=0.4$) are concerned, and only at the verge of significance with respect to the carbazole

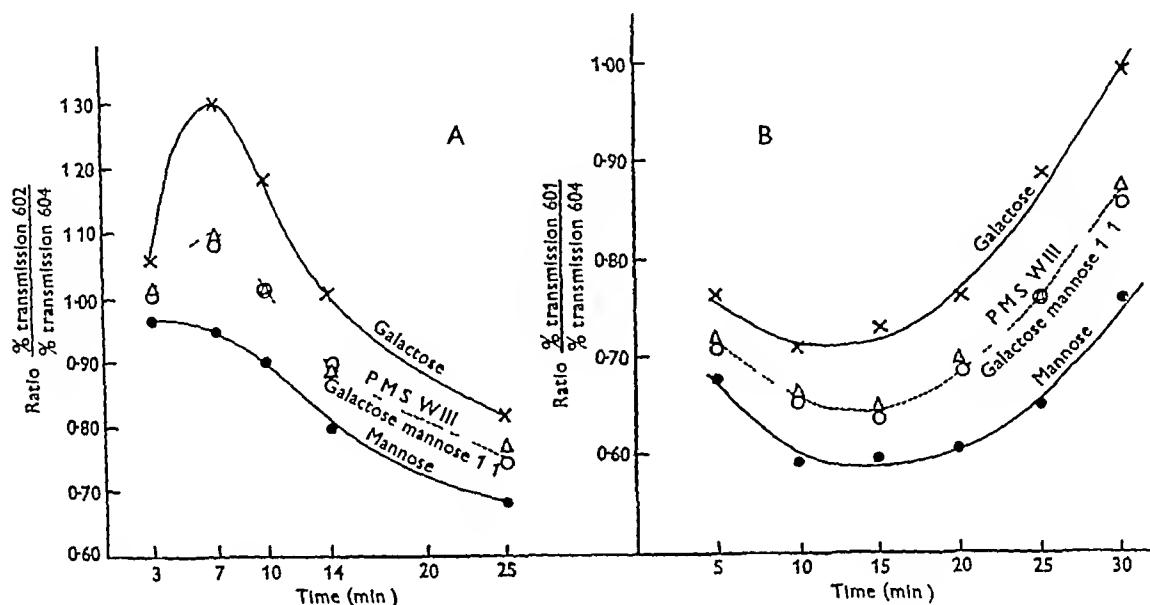


Fig 5 A comparison of the ratio time curves (see Figs 1 and 4) for mannose —●—, galactose —x—, an equimolar mixture of mannose and galactose ---⊙---, and a purified fraction of pregnant mares' serum (PMS W III) ---Δ---, as obtained with the orcinol method (A) and the skatole method (B)

pass unnoticed in the orcinol method in the presence of a mixture of mannose and galactose (see Fig 1 C), it would be obvious in the skatole method (Fig 4 C) and can also be excluded. In view of the distinction between mannose and galactose in the orcinol method, it is reasonably certain that the sugar component of PMS W III is not pure mannose or pure galactose.

The proteins of normal horse serum and of the sera of horses immunized against the toxins of *Corynebacterium diphtheriae*, *Clostridium perfringens* and *Cl. tetani* were purified by several precipitations with ammonium sulphate and finally dialyzed against running tap water to remove any residual uncombined carbohydrates. There was no difference either by the skatole, orcinol or carbazole method, in the ratio time curves of these sera, which were again consistent with an equimolar mannose-galactose composition.

The sugar component of the PMS sample was determined quantitatively 12 times by each of the three methods. An equimolar mixture of mannose and galactose was used for the standard curve. Table 4 shows that the accuracy and reproducibility is of the same order of magnitude for all three methods, but that the orcinol method gives a lower coefficient of variation than the other two methods.

Table 3 Carbazole method comparison of percentage transmission ratios obtained with a purified follicle-stimulating fraction of pregnant mares' serum (PMS W III), mannose, galactose and an equimolar mixture of mannose and galactose

Ilford spectrum filter ratio	604/601	604/602	607/604	607/601
Mannose	1.92	1.13	1.48	2.94
Mannose galactose = 1:1	1.55	1.04	1.56	2.50
PMS W III	1.60	1.03	1.53	2.48
Galactose	1.27	0.93	1.58	2.00

Table 4 Statistical comparison of the three methods

(Twelve estimations of the sugar component of a follicle stimulating fraction of pregnant mares' serum by each method.)

Method	Mean mg hexose/g protein	Standard deviation	Coefficient of variation (%)	Standard error
Orcinol	41.8	1.16	2.77	0.34
Carbazole	43.2	1.62	3.74	0.47
Skatole	42.3	1.67	3.95	0.49

$$P_{\text{orcinol carbazole}} = 0.04$$

$$P_{\text{orcinol skatole}} = 0.4$$

$$P_{\text{skatole orcinol}} = 0.2$$

and orcinol methods ($P = 0.04$). There is no significant difference between the figures obtained by the carbazole and skatole methods ($P = 0.2$).

DISCUSSION

A large number of nitrogenous and phenolic compounds form distinct coloured products with carbohydrates. The reactions with orcinol and carbazole have been studied in detail (Sørensen & Haugaard, 1933; Gurin & Hood, 1939), both methods have been applied widely, but have also been subject to many criticisms. We have now added the skatole method as a third alternative. All the methods suffer from lack of precision and lack of specificity, unless rigidly standardized. Small variations in the concentration of the acid and the colour-forming reagent or minute amounts of impurities influence the intensity and tint of the resulting colour. A variety of carbonyl compounds other than carbohydrates also give intensely coloured products with these reagents (Table 5). The significance of all colour tests for the qualitative characterization of sugars has been questioned (Meyer, 1938, 1945).

Table 5 *Colour reactions of carbonyl compounds other than carbohydrates with orcinol, carbazole and skatole*

	(Concentration of carbonyl compound 1 mg/ml)		
	Orcinol	Carbazole	Skatole
Formaldehyde	Bright orange	Deep blue precipitate	Dark brown precipitate
Benzaldehyde	Yellowish white precipitate	Raspberry red	Deep red
Vanillin	Blood red	Faint mauve	Deep clear ruby red
Acetone	Like blank	Like blank	Like blank
Blank	Nearly colourless	Faint grey	Faint pink

The present investigation shows that quantitative estimations are less subject to variation than qualitative characterizations, absolute quantitative figures may be obtained quickly and accurately (Table 4) provided the qualitative composition of the carbohydrate complex is known.

Qualitative characterization is possible within limits by adhering precisely to the given details of the methods and making use of all three methods in the analysis. The orcinol method distinguishes best between fructose and galactose (Fig 1 C). Less than 20% galactose cannot be detected in the presence of 80% mannose and vice versa (Fig 6). Only 1:1 mixtures of glucose and mannose may be resolved. With respect to qualitative characterization, the carbazole method has the attraction of greater speed, but there is less differentiation than in the orcinol and skatole methods, an equivalent mixture of

glucose and mannose cannot be distinguished from galactose (Gurin & Hood, 1939), and only 1:1 mixtures of any two sugars can be resolved (Table 6). The skatole method distinguishes best between glucose and mannose where admixtures of less than 20% are recognizable. An equimolar mixture of glucose and mannose cannot easily be differentiated from galactose (see Fig 4 C). It will be remembered that fructose is readily distinguished from the aldoses.

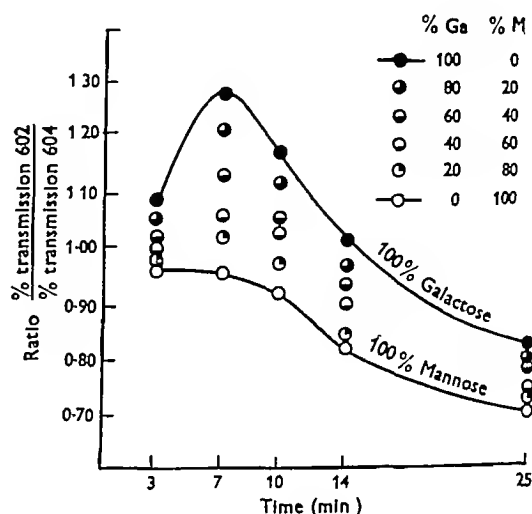


Fig 6 *Orcinol method. Characterization of mannose and galactose in the presence of each other by means of ratio time curves (see Fig 1) —●— galactose alone, ● 80% galactose + 20% mannose, ● 60% galactose + 40% mannose, ● 40% galactose + 60% mannose, ● 20% galactose + 80% mannose, —○— mannose alone*

Quantitative estimations are quickest with the orcinol method, since no special care needs to be taken over the initial mixing of the reagents, a critical point in the carbazole and skatole methods.

Table 6 *Carbazole method. Comparison of the percentage transmission ratios obtained for mannose, galactose and mixtures of the two sugars*

Ilford spectrum filter ratio	604 601	604 602	607 604	607 601
Mannose (M)	1.89	1.15	1.48	2.85
M Ga=80 40	1.67	1.07	1.48	2.46
M Ga=1 1	1.52	1.00	1.51	2.40
M Ga=20 80	1.34	0.97	1.57	2.04
Galactose (Ga)	1.22	0.93	1.57	1.92

Holzman, Macalister & Niemann (1947), using pure sugar solutions, have investigated the variables in the carbazole reaction. Following Seibert & Atno (1946), they added the carbazole in the sulphuric acid reagent, with better control of the amount of carbazole added, which they showed to be critical. However, the new reagent is not stable, and the precision of the modified procedure is the same as that of the original method (2–5% in pure sugar

solutions) Protein analyses made by Seibert & Atno (1946) using Gurin & Hood's (1939) carbazole method show variations of 5-6 % (three estimations), while we find a coefficient of variation of nearly 4 % (Table 4, twelve estimations) Staub & Rimington (1948) state that results obtained by the carbazole method are higher than those with orcinol, which appears to be borne out by our analysis, although the statistical significance of this difference is doubtful ($P=0.04$) Under the given precise conditions, the orcinol method gives more consistent results than the carbazole and skatole methods (coefficient of variation of 2.77 %, compared with 3.74 and 3.95 % respectively) and there is very little interference by protein (Table 1) Apart from any specific amino-acid interference, the high reaction temperature (carbazole method) and the high acid concentration lead in the carbazole and skatole methods to the production of more coloured decomposition products than can develop under the conditions of the orcinol reaction

The skatole method is perhaps the most satisfactory qualitative method, since it combines the possibilities of the orcinol and carbazole methods for characterization, but it unfortunately also shares the disadvantages of both procedures Fructose, although readily recognized, cannot be estimated quantitatively by this method in the presence of protein

Some comment is needed on the nature of the sugar component of P M S, since our findings are at variance with those of Li, Evans & Wonder (1940) and Gurin (1942) Li *et al.*, using a modification of the orcinol method, claim that the carbohydrate component other than glucosamine is galactose, while Gurin's results with the carbazole method indicate galactose or an equimolar mixture of glucose and mannose Rimington & Rowlands (1941, 1943, 1944), using the orcinol method, chose an equimolar mixture of mannose and galactose as the basis of the hexose estimations throughout their work on P M S, because the tints obtained with this mixture correspond closely to those obtained with P M S preparations Our more detailed investigations of this particular point by means of three different methods confirm their findings

Summing up, it can be said that the main value of the colour reactions investigated lies in the following

(1) To find quickly and accurately quantitative differences in the sugar contents when concentrating natural products The nature of the sugar does not matter then as one of the fractions can serve as an arbitrary standard, by means of time curves it will be possible to decide whether any significant changes in the qualitative composition of the sugar component—whatever its nature—take place during concentration

(2) To find quickly and accurately the absolute concentration of any mixture of sugars—however complicated—alone or in combination with protein, if the nature of the sugar component is known or the complex can be isolated

(3) To give a rough indication of the sugars present This may be a tedious process and it is almost impossible to analyze a mixture of more than two components The proportions of the components can only be ascertained approximately and it is important to have some idea of the composition beforehand The carbazole method appears less suitable for this purpose than the other two methods

SUMMARY

1 Compounds of two types, either of a phenolic character or containing a reactive nitrogen group, have been investigated for colour formation with four representative hexoses resulting in the development of the skatole method for the qualitative and quantitative characterization of sugars

2 The relative limitations of the orcinol, carbazole and skatole methods are compared and the necessary standardizations pointed out

3 All three methods have been applied to the analysis of the sugar component of a fraction of pregnant mares' serum The results obtained are consistent with an equimolar mannose-galactose composition (apart from glucosamine which is not estimated by the reactions used)

4 The sugar component of the blood proteins of normal and immunized horses have been analyzed similarly, and the results are again consistent with an equimolar ratio of mannose and galactose

It is my pleasant duty to thank Dr G E Foster of The Wellcome Chemical Works, Dartford, for his kindness in giving me hospitality in his laboratory, where the curves for Fig 2 were prepared

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Fractionation of Oxidized Insulin

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From the determination of the terminal residues of insulin it was suggested that the submolecule of molecular weight 12,000 is made up of four open peptide chains bound together by disulphide linkages (Sanger, 1945). Two of these chains have glycine and two have phenylalanine as terminal residues. By the action of performic acid it is possible to split insulin into its separate polypeptide chains by conversion of the cystine to cysteic acid residues (Sanger, 1947). The object of the present work was to separate the chains in a pure form, and the first problem was to find a suitable analytical method to follow the course of fractionation. Electrophoretic analysis gave rather variable results, this was largely due to the low molecular weight of the fractions which led to rapid spreading of the boundary by diffusion and did not allow preliminary dialysis. Later the method of end-group assay (Sanger, 1945, Porter & Sanger, 1948) was used in an attempt to obtain fractions containing only glycine or only phenylalanine terminal residues. This method of characterization does not necessarily indicate complete homogeneity, but the fractions are at least representative of the two types of chains in the insulin molecule. In this paper the preparation and some preliminary observations on the properties of two fractions are reported, the one, fraction A, containing only glycine terminal residues and no basic amino-acids, the other, fraction B, containing 97% phenylalanine terminal residues.

EXPERIMENTAL

Oxidation of insulin

The method was essentially that of Toennies & Homiller (1942), who showed that within 1 hr. cystine consumes the theoretical 5 atoms of oxygen, and that after 2 hr. a more general oxidation of a number of amino acids takes place. It was thus desirable to use a time of oxidation which was the minimum required to split the S-S bridges completely.

Using paper chromatography (Consden, Gordon & Martin, 1946b) it was found that the reaction with free cystine was complete in 5 min., but with insulin 15 min. were required. The method finally used was as follows: 0.25 g. insulin was dissolved in 9 ml. formic acid, 1 ml. 30% (w/w) H_2O_2 was added, and the mixture allowed to stand for 15 min. at room temperature. Water (10 ml.) was added and the mixture evaporated *in vacuo* to a small volume (1–2 ml.). The oxidized protein was then precipitated by a large volume of acetone, centrifuged, washed with acetone till free of formic acid and dried in air, yield, 0.25 g.

Fractionation of the oxidized insulin

Of the various fractionation procedures tried, the most satisfactory were the conventional methods of precipitation by alteration of pH or addition of salt or ethanol. In the salting-out procedures, however, it was difficult to remove the salt since the peptides were too small to be dialyzed. This was overcome by using a volatile salt, ammonium acetate, which could be removed by evaporation.

Freshly oxidized insulin (1 g.) was dissolved in 12 ml. 0.1M- NH_3 , and 0.1M acetic acid was added to bring the pH of the solution to 6.5. The precipitate was centrifuged down and used in the preparation of fraction B. The solution was used for the preparation of fraction A.

Fraction A. The above solution was brought to pH 4.5 with 0.1M acetic acid and, after removal of the precipitate (fraction M), was taken almost to dryness *in vacuo*. It was then transferred to a centrifuge tube with a minimum volume (about 3 ml.) of water, and an equal volume of 50% (w/v) ammonium acetate, which had been brought to pH 5.5 with glacial acetic acid, was added. This brought about the separation of a small precipitate which was discarded. The water and ammonium acetate were then removed by leaving the solution in a high vacuum over H_2SO_4 and NaOH till it reached constant weight. The residue was a white powder, yield, 0.25–0.32 g.

Fraction B. The material precipitated at pH 6.5 from 1 g. oxidized insulin was washed with 10 ml. 0.01M acetic acid, dissolved in 5 ml. 0.1M-HCl and 40 ml. absolute ethanol added. This brought about the separation of a precipitate (fraction X) often in the form of a gel, which was centrifuged off and washed well with 80% ethanol. The combined

supernatant solution and washings were taken almost to dryness *in vacuo* and precipitated by a large volume of acetone, yield, 0.14–0.21 g. The yield of fraction X was 0.1–0.2 g. Various other methods were studied as a means of fractionating the oxidized insulin, but none was found more suitable than the above procedure.

Fraction A could be prepared satisfactorily by ionophoresis. Using ammonium acetate buffer, according to method C1 of Consden, Gordon & Martin (1946a), it moved towards the anode as a discrete band, and could be identified by applying the Pauly diazo reaction to a print. After removal of the ammonium acetate *in vacuo* and elution with water the fraction was shown to contain only glycine terminal residues. Fraction B moved only slowly at various pH values and formed badly tailing bands indicating that adsorption on the gel had taken place. It was difficult to elute from the gel, and only a small yield was obtained of material containing only phenylalanine terminal residues.

The method of adsorption analysis (Tiselius, 1947) could be used as an analytical method (Tiselius & Sanger, 1947), but it was not found possible to use elution or displacement analysis to fractionate the material.

Using paper chromatography (Consden, Gordon & Martin, 1944), it was difficult to find a sufficiently sensitive colour test that could be applied to the paper. A weakly positive ninhydrin test was given by fraction B, as by insulin, but was not given by fraction A. The most satisfactory test was the Pauly reaction, which is given by histidine and tyrosine residues. Using this test and relatively high concentrations of the peptides it was found that they formed fast moving tailing spots with phenol and collidine, and did not move with butanol or butanol acetic acid.

Properties of the fractions

Fraction A Fraction A is the most soluble material in the oxidized insulin. It is not precipitated at any pH value in low salt concentrations, or by any concentrations of sodium chloride or ammonium acetate, but it can be precipitated by high concentrations of ammonium sulphate.

The material, prepared as described above, contained 2.12% NH_2 bound in salt linkage presumably to the sulphonic acid groups. Excluding this N, the N content of the dry protein was 12.80% and the amide N 15.15% of the total N. End-group analysis showed almost only glycine and less than 1% phenylalanine terminal residues. The 2,4-dinitrophenyl (DNP) derivative which was prepared in the usual manner was slightly soluble in acid. Excess 1,2,4-fluorodinitrobenzene was removed as follows. The reaction mixture was taken to a small volume *in vacuo*, and the residue suspended in water and extracted well with ether. On acidification the DNP derivative was partly precipitated. Both the precipitate and the solution yielded only DNP-glycine on hydrolysis.

Paper chromatography (Consden *et al.* 1944) of a hydrolysate showed the following amino acids to be present: leucine, isoleucine, valine, tyrosine, alanine, glycine, serine and glutamic, aspartic and cysteic

acids. The following, though present in insulin, were absent from fraction A: lysine, arginine, histidine, threonine and phenylalanine. No proline spot could be detected, but its absence is not absolutely certain, as the ninhydrin test is not so sensitive as with other amino-acids. The absence of arginine and histidine was confirmed by the methods of Macpherson (1946) and of lysine by the absence of N^5 -DNP-lysine in a hydrolysate of the DNP derivative. The absence of threonine was demonstrated by Mr M. W. Rees using the periodate technique (Rees, 1946). A very rough analysis was carried out by a method similar to the 'spot-dilution' technique of Polson, Mosley & Wyckoff (1947). All the amino-acids present could be separated using phenol-0.3% NH_3 -coal gas as solvent, standard solutions of amino-acids were run parallel with the hydrolysate of fraction A and the colour and size of the spots compared. The results are shown in Table 1. They are expressed as the number of residues of amino-acids/mol of mol wt 2500, assuming fraction A to be homogeneous. While this is probably not true, the figures do give an approximate estimate of the amino acid distribution within the limits of the method.

Table 1 *Amino-acid composition of fraction A*

Amino acid	No of residues/mol of mol wt 2500	Amino acid	No of residues/mol of mol wt 2500
Leucine	3	Glycine	1
Isoleucine	1	Serine	2
Valine	2	Glutamic acid	4
Tyrosine	2	Aspartic acid	2
Alanine	1	Cysteic acid	4

In developing a new method for the determination of sedimentation constants of substances of low molecular weight in the ultracentrifuge, Gutfreund & Ogston (1948) studied a rather cruder preparation of fraction A in which the ammonium acetate precipitation was omitted. They considered it to be relatively homogeneous and to have a mol wt of 2900.

Fraction B Fraction B is readily precipitated near pH 6 and also by low concentrations of salt. Thus a solution in 0.03 M-HCl is precipitated by 1.5% NaCl. Electrophoretic analysis at pH 8 showed one main boundary and a trace of a faster moving component. On ionophoresis at pH 7 it appeared to be slightly more basic than unchanged insulin, in spite of its cysteic acid content, suggesting a high content of basic amino-acids.

End-group analysis showed about 97% phenylalanine and about 3% glycine terminal residues. It has not yet been possible to obtain a preparation completely free of glycine terminal residues. Paper chromatography showed the presence of all the amino-acids that are found in insulin, that is to say,

all the commonly occurring amino-acids except tryptophan, methionine and hydroxyproline

In the ultracentrifuge (Gutfreund & Ogston, 1948) this preparation appeared to be less homogeneous than fraction *A* and to have a mean mol wt 7000

DISCUSSION

By a variety of methods, including precipitation at pH 6 or with 3–30 % NaCl or ionophoresis at pH 7, it is possible to separate the oxidized insulin into two crude fractions: an acidic fraction *A*, with glycine as terminal residues and a basic fraction *B*, with phenylalanine as terminal residues. This does suggest that there are essentially two types of peptide chains in insulin, although the different chains of each type are probably not identical. Since the yield of the pure fraction *A* is usually greater than 25 % of the oxidized insulin, it follows that, if there are two glyceryl chains in insulin, then they must both be present in this fraction, so that, besides having the same terminal residue and no basic amino acids, they must also both be free from threonine and phenylalanine and have the same molecular weight and electrophoretic mobility.

Besides fractions *A* and *B*, the only other significant fractions are fractions *X* and *M*. It seems likely that both of the latter contain essentially the same material, since by using a lower pH for the initial precipitation, the yield of fraction *X* is increased at the expense of fraction *M* and vice versa. The sum of the yields of the two fractions is 20–30 % of the oxidized insulin, and they contain about equal

amounts of the two terminal residues. By repeating the ethanol precipitation on either *X* or *M* a certain amount of fraction *B* can be prepared from them, but it seems probable that they consist chiefly of a mixture of incompletely oxidized and over oxidized insulin. Fraction *X* tends to darken on standing, suggesting the presence of oxidation products of tyrosine. The yield of these fractions is greater if an oxidation time of 5 min is used instead of 15 min, although it is not possible to lower the yield by further oxidation. While the possibility cannot be excluded that these fractions contain another chain of the insulin molecule, it seems likely that all the chains are actually represented in the purified fractions *A* and *B*. Thus it appears that the insulin submolecule is built up of two types of peptide chains, a basic type and an acidic type. It is possible that this particular structure may account for some of the properties of insulin.

SUMMARY

Two fractions have been prepared from insulin that has been oxidized by performic acid.

A, an acidic fraction containing glycine as terminal residues and no arginine, histidine, lysine, phenylalanine or threonine.

B, a basic fraction containing phenylalanine as terminal residues and all the amino acids present in insulin.

I wish to express my thanks to Mr M. W. Rees for the threonine analysis and to Prof. A. C. Chibnall for his advice and encouragement.

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The Formation of Hydrogen Carriers by Haematin-Catalyzed Peroxidations

1 HYDROGEN CARRIERS FROM CERTAIN ACRIDINE AND QUINOLINE COMPOUNDS

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During studies of the inhibition of enzymes by anti-bacterial acridines, it was found that certain of these drugs caused a large stimulation of oxygen uptake in solutions containing ascorbic acid, cytochrome *c* and cytochrome oxidase. The same oxygen uptake occurred when the oxidase was omitted, and hence this enzyme played no part in the reaction. The studies which follow were, therefore, made in the absence of cytochrome oxidase.

Because the new effect interfered with the desired measurement of enzyme inhibitions, and, because it had possible importance in regard to the biological action of acridine compounds, its mechanism was studied in some detail. The effect occurred whenever certain amino- or hydroxy-acridines or analogous quinolines were shaken with ascorbic acid, cytochrome *c* and air. Most of the detailed studies which follow were made with 3-aminoacridine.

A brief outline of the apparent mechanism is given here. The new effect is characterized by a primary phase during which the rate of oxygen uptake increases to a maximum, and a secondary phase during which this maximum rate is maintained until the substrate is exhausted, whereupon the uptake of oxygen ceases. The initiation of the effect depends on a trace of hydrogen peroxide (the origin of which is discussed below) which, in the presence of cytochrome *c*, brings about a catalyzed peroxidative oxidation. For example, 3-aminoacridine is oxidized to an *o*-hydroxyaminoacridine which is, in turn, autooxidizable to an *o*-quinonimine with production of hydrogen peroxide. By utilization of this hydrogen peroxide in the peroxidative reaction the effect becomes autocatalytic. The *o*-quinonimine acts as a hydrogen carrier in the oxidation of the substrate and oxygen is the ultimate hydrogen acceptor.

A similar series of reactions was found to occur when cytochrome *c* was replaced by methaemoglobin so that the initial reaction is evidently a nonspecific haematin peroxidative effect. Similar effects were found with cysteine as substrate but not with hydroquinone. There was no relation between the antibacterial activity of the acridine and quinoline compounds and their ability to give the effect.

EXPERIMENTAL

Materials Ascorbic acid was used as the pure substance, kindly supplied by the Colonial Sugar Refining Co., Sydney. It was determined by titration with 2,6-dichlorophenolindophenol (cf. Lemberg, Cortis Jones & Norrie, 1938). Cysteine was the Schering Kahlbaum copper-free preparation. Hydrogen peroxide (100 vol) was freshly diluted before use and standardized with 0.01N KMnO_4 . Water for all purposes was double distilled from an all glass still. The catalase was a preparation from horse liver, with a *Kat f* of 25,000. The cytochrome *c* contained 0.33% Fe and was obtained from ox heart by method (a) of Keilin & Hartree (1945). It was dialyzed against distilled water in Tee Pak synthetic sausage casings (Harvey Little and Co., Sydney) which adsorbed very little cytochrome. The catalase-free methaemoglobin solution was prepared as follows. A solution of pure crystalline haemin in dilute Na_2CO_3 was accurately determined spectrophotometrically as pyridine haemochromogen. A solution of native human globin was prepared by the method of Anson & Mirsky (1925) and its protein content determined. The solutions of haemin and globin were mixed so that there was 10% excess of globin. For the manometric experiments a volume of this solution equivalent to 1.0 mg of methaemoglobin was used. In terms of Fe content, this was equivalent to 1 mg of cytochrome *c*.

Aniline (b.p. 184°) and phenol (b.p. 182°) were freshly distilled. The m.p. of the *o*-phenylenediamine was 103° , *p*-phenylenediamine, 139° , catechol, 104° , *o*-aminophenol, 170° and *p*-aminophenol, 184° . The preparations and m.p.'s of the acridine compounds listed in Table 3 were as given by Albert, Rubbo, Goldacre, Davey & Stone (1945), and of the quinoline compounds as given by Albert & Magrath (1947) with the following additions. 3,5-dihydroxyacridine (m.p. $347\text{--}348^\circ$) was prepared according to Ullmann & Kipper (1907), 5-aminoquinoline (m.p. 110°) by the hydrogenation of 5-nitroquinoline over Raney nickel, 5-hydroxyquinoline (m.p. 223°) by the method of Claus & Howitz (1893) and 5-amino-6-hydroxyquinoline (m.p. 195° , decomp.) by the method of Mathéus (1888). Phenol blue, *p*-dimethylaminoaniline hydrochloride and *p*-diethylaminoaniline hydrochloride were obtained from British Drug Houses Ltd.

Methods The measurements of O_2 uptake were made in Warburg respirometers at pH 7.0 and 25 or 37.5° . The shaking rate was 120/min, the gas phase usually air, and, in the total liquid volume of 2.0 ml., the phosphate buffer was 0.12M, changing the buffer concentration did not affect the results. Experimental pH values were checked with a glass

electrode All solutions were prepared just prior to use The basic compounds were dissolved by the aid of 1-1.5 equiv of HCl, and the hydroxy compounds, when necessary, with 1 equiv of NaOH The ascorbic acid was dissolved in water, with the addition of 1 equiv of 0.1N NaOH, just before pipetting

RESULTS

The effect

The nature of the effect is summarized in Fig 1, curve 1 showing the typical autocatalytic reaction When the ascorbic acid concentration was halved, the total oxygen uptake was also approximately halved (curve 2)

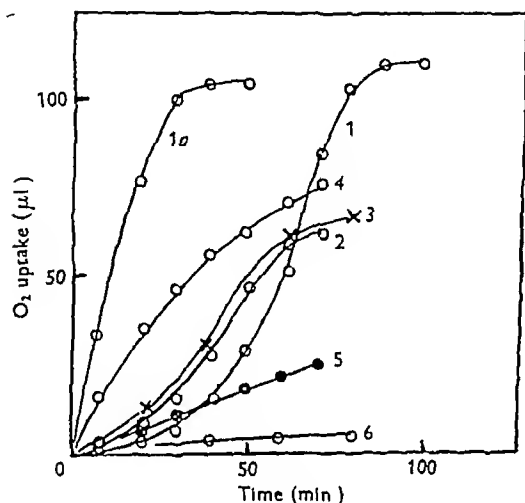


Fig 1 The effect obtained with 3-aminoacridine, sodium ascorbate and cytochrome *c* at pH 7.0 and 25°. Curve 1 3-aminoacridine, ascorbate and cytochrome *c* (concentrations in Table 1) Curve 1a uptake on addition of a second lot of ascorbate after reaction 1 Curve 2 as curve 1 with ascorbate concentration halved Curve 3 the rate of utilization of ascorbate determined by titration (see text) Curve 4 80% of the precipitate from reaction 1, isolated as described in text, and allowed to react with ascorbate only, addition of cytochrome *c* made no difference Curve 5 autooxidation controls, with ascorbate Curve 6 autooxidation controls without ascorbate

When the reaction was stopped at successive time intervals by the addition of trichloroacetic acid, and the amount of ascorbic acid used determined by titration, the rate of its utilization was parallel to the rate of oxygen uptake, in curve 3 this rate is plotted as the calculated amount of oxygen required to oxidize (to dehydroascorbic acid) the ascorbic acid used up in the given times The calculated amount of oxygen required to oxidize all the ascorbic acid to dehydroascorbic acid was 125 μ l, and the total uptake in curve 1 shows that it was not oxidized past this stage In a small number of experiments with cysteine as substrate, a similar effect was found, the total oxygen uptake then being only slightly

more than that required to produce cystine Hydroquinone gave no oxygen uptake at all, although it is a substrate for the full cytochrome *c* cytochrome oxidase system

When the effect occurred, a dark coloured precipitate was usually observed at the end of the reaction This precipitate dissolved, with loss of the dark colour, on addition of further ascorbic acid, and reappeared when this had been oxidized It was similarly dissolved by the addition of cysteine or sodium dithionite (hydrosulphite) The precipitate, isolated by centrifuging and washed well at the centrifuge with cold phosphate buffer, pH 7.0, catalyzed the oxidation of ascorbic acid by oxygen (curve 4) Addition of cytochrome *c* made no difference to the rate, unless some unchanged test substance was added with an incompletely washed precipitate, in which case cytochrome *c* caused a significant increase The precipitate lost its catalytic activity entirely when kept in the buffer for 2 days at 0° Its colour, its behaviour on reduction and re-oxidation and its instability strongly suggested a substance of quinonoid type

Table 1 The effect of varying the concentration of 3-aminoacridine and of cytochrome *c*, at pH 7.0 and 25°

(Each flask contained hydrogen peroxide, $10^{-3}M$, sodium diethyldithiocarbamate $6.4 \times 10^{-3}M$, and sodium ascorbate $5.6 \times 10^{-3}M$, as well as, in experiment (a), cytochrome *c* $2 \times 10^{-5}M$ and, in experiment (b), 3-aminoacridine $10^{-3}M$)

(a)	
3-Aminoacridine M	O ₂ uptake in 30 min (μ l)
0	0
10^{-5}	12
10^{-4}	52
10^{-3}	98
(b)	
Cytochrome <i>c</i> M $\times 10^{-5}$	
0	0
0.18	14
0.54	47
0.90	79
1.80	105

It was important to determine whether the precipitate contained cytochrome *c* or a derivative, e.g. an autooxidizable haem compound No absorption band whatever could be seen in a suspension of well washed, active precipitate, or in a solution made by reducing it with sodium dithionite Nor could iron be detected in the precipitate after digestion with concentrated sulphuric acid and hydrogen peroxide If the precipitate were a compound or mixture of 3-aminoacridine and cytochrome *c* in molecular ratios 100:1, iron could readily have been detected under the conditions of the test The precipitate must, therefore, be an iron-free derivative of 3-aminoacridine

Studies of the mechanism

In the presence of either cytochrome *c* or met-haemoglobin, the effect was inhibited by catalase on the one hand (Table 2), and stimulated by hydrogen peroxide on the other (Fig 2) In the absence of a haematin compound the effect did not occur, and was not induced by hydrogen peroxide (Table 2) There was thus strong evidence for the occurrence of a haematin catalyzed peroxidative reaction

Under certain conditions, either the 3-amino-acridine concentration or the cytochrome *c* concentration could limit the rate of reaction (Table 1) In these experiments hydrogen peroxide was added so that there was no lag, and the initial rates were comparable

Cytochrome *c* does not combine with carbon monoxide, but haemoglobin formed by reduction of methaemoglobin by ascorbic acid, combines with carbon monoxide to form carboxyhaemoglobin in which the iron is fixed in the reduced state In a gas phase of 80 % CO + 20 % O₂, the rates were only slightly lower than when the gas phase was air A similar slight CO inhibition was found when cytochrome *c* was the catalyst (Table 2) Carbon monoxide is known to inhibit the copper catalyzed autoxidation of ascorbic acid (Barron, DeMeio & Klemperer, 1936), and in our control experiments autoxidation was inhibited by carbon monoxide as well as by diethyldithiocarbamate The latter reagent also slightly depressed the effect in the presence of cytochrome *c* (Table 2) The autoxidation of ascorbic acid produces hydrogen peroxide, and the CO and diethyldithiocarbamate inhibitions of the effect may be due to their prevention of the formation

of this hydrogen peroxide If this was a limiting factor, the rate during the autocatalytic stage would be depressed and the lag lengthened This was

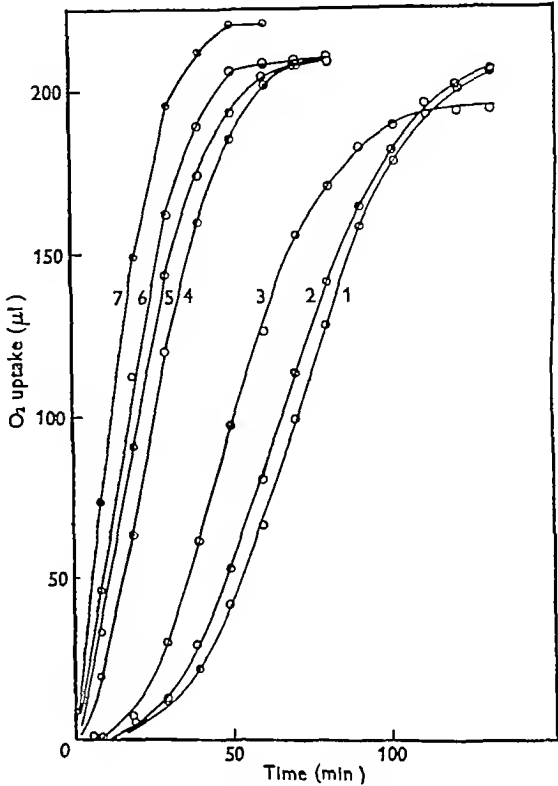


Fig 2 The effect obtained with 3 aminoacridine and different concentrations of hydrogen peroxide at pH 7.0 and 25° Each flask contained 3 aminoacridine, cytochrome *c* and ascorbate with concentrations as in Table 1 Curves 1-7 hydrogen peroxide ml, 0.01, 0.1, 0.25, 0.5, 1.0 and 10.0 × 10⁻⁴ M respectively

Table 2 The effect of various reagents on the rate of oxygen uptake of ascorbic acid with 3 aminoacridine at pH 7 and 37.5°

(Each flask contained, in separate side arms, sodium ascorbate (final molarity 5.6 × 10⁻³) and 3 aminoacridine (final molarity 2 × 10⁻³) These were tipped on to the reagents indicated in column 1 after equilibration Molarity of cytochrome *c*, 0.9 × 10⁻⁵, of methaemoglobin, 0.22 × 10⁻⁵, of H₂O₂, 1.0 × 10⁻³, of CuSO₄, 2.0 × 10⁻⁵, of sodium diethyldithiocarbamate 6.4 × 10⁻³ Values of *t* (time to reach maximum rate) were accurately reproduced to ± 3 min, and of *v* (the maximum rate) to at least ± 0.5 μl/min, accuracy increasing as *v* became smaller)

Added to main bulb	Gas phase	<i>t</i> (min)	<i>v</i> (μl O ₂ /min)
Nil	Air	0	0.5
Nil	CO O ₂	0	0
Diethyldithiocarbamate	Air	0	0
Cytochrome <i>c</i>	Air	35	5.5
Cytochrome <i>c</i>	CO O ₂	55	3.0
Cytochrome <i>c</i> + H ₂ O ₂	Air	0	18.3
Cytochrome <i>c</i> + catalase	Air	65	2.0
Cytochrome <i>c</i> + diethyldithiocarbamate	Air	43	5.0
Cytochrome <i>c</i> + CuSO ₄	Air	0	5.0
Cytochrome <i>c</i> + CuSO ₄	CO O ₂	0	4.0
Methaemoglobin	Air	45	3.6
Methaemoglobin	CO O ₂	60	2.9
Methaemoglobin + H ₂ O ₂	Air	0	18.0
Methaemoglobin + catalase	Air	0	0.6

indirectly confirmed by addition of small amounts of copper sulphate. As shown in Table 2, the lag was abolished, in the presence of copper the reaction was again inhibited to some extent by carbon monoxide.

Although hydrogen peroxide could arise from autooxidation of the ascorbic acid, it was realized that this was not necessarily the source of the peroxide which initiated the effect. The effect still occurred in the presence of carbon monoxide or diethyldithiocarbamate, although autooxidation was then negligible. In addition, when the 3-aminoacridine was shaken with the cytochrome *c* for 30 min before adding the ascorbic acid, considerable darkening in colour had occurred, and the lag was shorter than when the reagents were mixed immediately after equilibration as was usual. This darkening was inhibited by catalase.

This formation of carrier in the absence of ascorbic acid suggested that a trace of hydrogen peroxide was introduced from another source. It was found that the cytochrome *c* solutions, prepared as above, gave an output of oxygen gas on the addition of catalase. It is not yet known at what stage of the preparation this peroxide arose, and, in view of the importance of the presence of this unsuspected contaminant in preparations of cytochrome *c* made in accordance with published instructions, this question is now under investigation.

The chemical change in the peroxidative reaction

The following (I, II, III) are the reversible hydrogen carrying systems which appear most likely to be formed by oxidation of 3-aminoacridine.

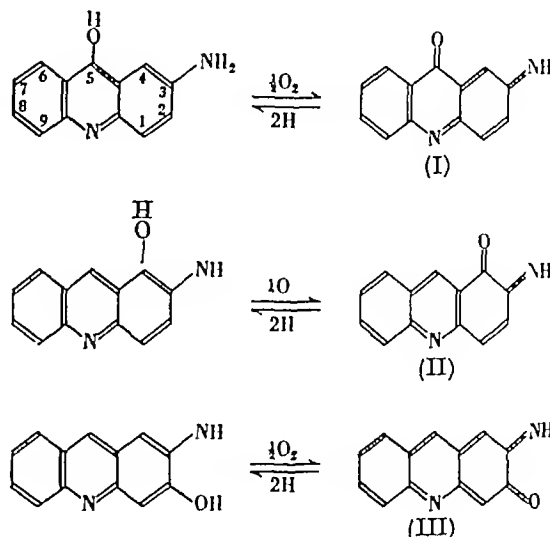
In order to explore these possibilities, it was decided to examine the series of acridine and quinoline compounds given in Table 3. The ionization (as bases) under the conditions of the experiment have been included in the table as this was found to be relevant. The antibacterial activities of these compounds are also recorded to show that the well-known antibacterial properties of some members of these series do not run parallel to their ability to become carriers.

The results obtained from the acridine compounds are given in Table 4 and those from the quinoline compounds in Table 5.

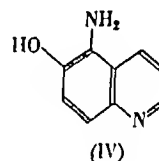
Compounds of the types (I-III) could be formed from any of the acridine compounds which gave the effect (Table 4), quinones would be the corresponding oxidized forms of the carriers derived from hydroxyacridines.

Carriers of type (I) were excluded, however, since neither 3-amino-5-hydroxyacridine nor 3,5-dihydroxyacridine (nos 15 and 16) had any carrier activity. Oxidation in the position ortho to the existing amino or hydroxyl group thus seemed more likely.

Since acridine compounds of types (II) and (III) are unknown and likely to be extremely difficult of access, attention was concentrated on the quinoline

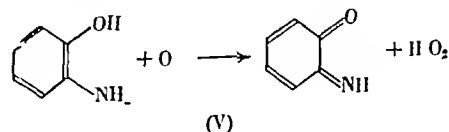


compounds (Table 5). From 5-aminoquinoline, which gave an effect similar to 3-aminoacridine, only one ortho-substituted compound (IV) is possible.



This compound, 5-amino-6-hydroxyquinoline, was synthesized, and was found to act directly as a reversible hydrogen carrier in the oxidation of ascorbic acid, 7-amino-8-hydroxyquinoline acted similarly, as also did the indamine dye, phenol blue.

There is thus good reason to believe that the carrier formed from 5-aminoquinoline is the readily autooxidizable substance, 5-amino-8-hydroxyquinoline. It became important to determine whether, as assumed above, autooxidation of compounds of this type gives rise to hydrogen peroxide (as in V).



When solutions in phosphate buffer, pH 7, of 5-amino-6-hydroxyquinoline and 7-amino-8-hydroxyquinoline were shaken with air at 25 or 37.5°, there was with the latter compound a fast uptake of oxygen, the full theoretical amount being consumed in about 1 hr. The former consumed oxygen more slowly, and, in 1 hr, only 75% of the theoretical amount had been taken up. When catalase was added after 1 hr, there was in each case an oxygen output

Table 3 *The acridine and quinoline compounds used, their strength as bases and their antibacterial activity (where known)*

No	Compound	pK _a , basic, 25° in water	Percentage ionized as base, 25° pH 7 (g)	B I (h)
1	Acridine	4.52 (a)	0.34	6
2	1 Aminoacridine	4.33 (a)	0.22	4
3	2 Aminoacridine	7.94 (a)	89.70	21
4	3 Aminoacridine	5.80 (a)	5.94	8
5	4 Aminoacridine	5.96 (a)	8.37	9
6	5 Aminoacridine	9.88 (a)	99.86	25
7	2,7 Diaminoacridine	8.02 (b)	91.2	26
8	2,8 Diaminoacridine	9.54 (b)	99.4	22
9	2,8 Diamino 1,9 dimethylacridine	9.05 (c)	99.11	30
10	2,8 Diamino 3,7 dimethylacridine	10.23 (c)	99.94	21
11	2,8 Bisdimethylaminoacridine	10.57 (c)	99.97	17
12	3 Amino 5 methylacridine	6.14 (c)	12.23	10
13	3 Hydroxyacridine	4.63 (d)	0.43	0
14	5 Hydroxyacridine (acridone)	—	<0.01 (d)	0
15	3,5 Dihydroxyacridine	—	<0.01	—
16	3-Amino 5 hydroxyacridine	—	—	—
17	5 Aminoquinoline	5.43 (e)	2.35	0
18	6 Aminoquinoline	5.54 (e)	3.36	0
19	5 Hydroxyquinoline	—	—	0
20	8 Hydroxyquinoline	4.95 (f)	0.95	26
21	5 Amino 6 hydroxyquinoline	—	—	—
22	7 Amino 8 hydroxyquinoline	—	—	—

(a) Calculated from values in water at 20° (Albert & Goldacre, 1946), by a temperature correction found by interpolation in a curve based on the data of Hall & Sprinkle (1932)

(b) Kindly determined for this work by Mr J. N. Phillips

(c) Calculated from values in 50% ethanol (Albert & Goldacre, 1946), by adding 0.64, the mean depression by ethanol for 11 compounds (Albert & Goldacre, 1946). Temperature correction as in (a) then applied

(d) Calculated from data of Albert & Goldacre (1943)

(e) Calculated from data of Albert & Goldacre (1944)

(f) Calculated from data of Albert & Magrath (1947)

(g) Calculated from the equation $\% \text{ ionized} = \frac{100}{1 + \text{antilog}(\text{pH} - \text{pK}_a)}$

(h) The Bacteriostatic Index (from Albert *et al.* 1945, Albert, Rubbo, Goldacre & Balfour, 1947). A B I of 15 indicates significant antibacterial activity, and a B I of 20 or higher, marked activity

Table 4 *The results of testing various acridine compounds*

(The compounds were tested with ascorbic acid and cytochrome *c*, with air as gas phase, with concentrations and conditions as in Table 1. Where the effect was found, the reactions when hydrogen peroxide or catalase was added were similar to those shown in Table 2. Hydrogen peroxide did not induce the effect in the compounds in column 2.)

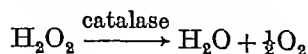
(1) Compounds which gave the effect at pH 7		(2) Compounds which were completely inactive	
No		No	
4	3 Aminoacridine	1	Acridine
5	4 Aminoacridine	2	1 Aminoacridine
7	2,7-Diaminoacridine	6	5 Aminoacridine
8	2,8 Diaminoacridine	10	2,8 Diamino 3,7 dimethylacridine
9	2,8 Diamino 1,9 dimethylacridine	11	2,8 Bisdimethylaminoacridine
13	3 Hydroxyacridine	12	3 Amino 5 methylacridine
3	2 Aminoacridine (at pH 8.5)	14	5 Hydroxyacridine (acridone)
		15	3,5 Dihydroxyacridine
		16	3 Amino 5 hydroxyacridine

Table 5 *The results of testing various quinoline compounds*

(The compounds in column 2 were directly active as carriers in the oxidation of ascorbic acid. The v values were for the O₂ uptake of $5.6 \times 10^{-3} \text{ M}$ ascorbic acid with the carrier $2 \times 10^{-3} \text{ M}$, at pH 7.0 and 25°, there was no lag, and addition of cytochrome *c* made no difference. Of these compounds, only nos. 18 and 22 gave a precipitate (see text).)

(1) Compounds which gave the effect		(2) Compounds which were active even in the absence of cytochrome <i>c</i>	
No		No	v ($\mu\text{l O}_2/\text{min}$)
17	5 Aminoquinoline	21	5 Amino 6 hydroxyquinoline
18	6 Aminoquinoline	22	7 Amino 8 hydroxyquinoline
19	5 Hydroxyquinoline		Phenol blue (for comparison)
20	8 Hydroxyquinoline		

corresponding to half the oxygen consumed as required by (V) and the equation,



That hydrogen peroxide is formed on autoxidation of these compounds at pH 7 was thus established. When *o*- and *p*-aminophenol were tested in the same way, however, no oxygen uptake or output was found with either.

The failure of some of the acridine compounds to give the effect must now be considered. The failure of 2-aminoacridine to yield any carrier in the usual test at pH 7 was surprising, in view of the activity of its derivatives, nos 7 and 8 (Table 4). 3-Aminoacridine at pH 7 is mainly un-ionized, while 2-aminoacridine is ionized to the extent of 89.7%. Although 2,8-diaminoacridine exists almost entirely in the cationic form at pH 7, it is likely that a significant proportion of one amino group remains un-ionized. In 2,7-diaminoacridine, the 7-amino group does not enter into the ionic resonance and is entirely un-ionized (Albert & Goldacre, 1946). It thus seemed possible that 2-aminoacridine might give the effect if it could be tested with a higher proportion of its amino group in the un-ionized form. This proved to be the case (Table 6). At pH 8.5, when it was 78% un-ionized, it gave a carrier effect similar to that of 3-aminoacridine.

Although, in this group of substances, the presence of an un-ionized amino group appears to be one of the prerequisites for carrier formation, other factors also operate. For example, at pH 7 no 2 is almost completely un-ionized but gives no carrier (Table 4).

A few benzenoid compounds were tested to find whether the effect would also occur in this series. The result was negative. Aniline, phenol, catechol, hydroquinone and *o*-phenylenediamine gave no carriers under any conditions. Phenol blue and 2,6-dichlorophenolindophenol acted directly as carriers in the well known manner, no cytochrome *c* being required. *p*-Phenylenediamine, *p*-dimethylaminoaniline, *p*-diethylaminoaniline and *p*-aminophenol all gave carriers which acted similarly to phenol blue in Table 5, provided that they were shaken with cytochrome *c* at 25° and pH 7 for 0.5 hr before adding the ascorbic acid. Under these conditions they were oxidized to coloured compounds, on

adding the ascorbic acid the colour disappeared, but returned when the ascorbic acid had been oxidized. In the case of the first three compounds it was probable that the carriers were the Wurster's salts which they are known to yield on oxidation. It seemed that ascorbic acid, if added too soon, protected them from oxidation to this form.

DISCUSSION

For the effect to occur, it is evidently necessary that (a) A peroxidative oxidation should take place in such a way that a hydroxyl group enters the acridine or quinoline nucleus ortho (or, conceivably, para) to an existing amino or hydroxyl group (b) The product of this reaction should be of suitable oxidation-reduction potential to act as a reversible hydrogen carrier in the oxidation of ascorbic acid by oxygen.

In Table 4, a number of compounds which did not give the effect are shown. Comparison of nos 4 and 12 is interesting, the 5-methyl group in no 12 should make little difference to its oxidizability ortho to the amino group (i.e. in the 2- or 4 positions), or to the oxidation-reduction potential of the oxidized compound, but it is possible that the methyl group sterically hindered the peroxidative reaction. Similar considerations apply to no 10 (compare no 9) and to nos 15 and 16 which have been discussed above. No 6 has no oxidizable position ortho to its amino group.

The 3-aminoacridine effect appears to depend on the mechanism shown in the scheme on p. 135.

In the case of the hydroxy compounds (e.g. 3-hydroxyacridine) the reduced form of the reversible system is probably an *o*-dihydric phenol, and the oxidized form an *o*-quinone.

Evidence has been found that solutions of cytochrome *c*, prepared by the method of Keilm & Hartree (1945), may contain small amounts of hydrogen peroxide. This could make a considerable difference to *in vitro* studies, for example by initiating coupled oxidations, such as have been described above. Work which is proceeding on this subject will be reported separately.

Some examples will now be mentioned in which previous workers seem to have met the effect reported here without having recognized its true nature.

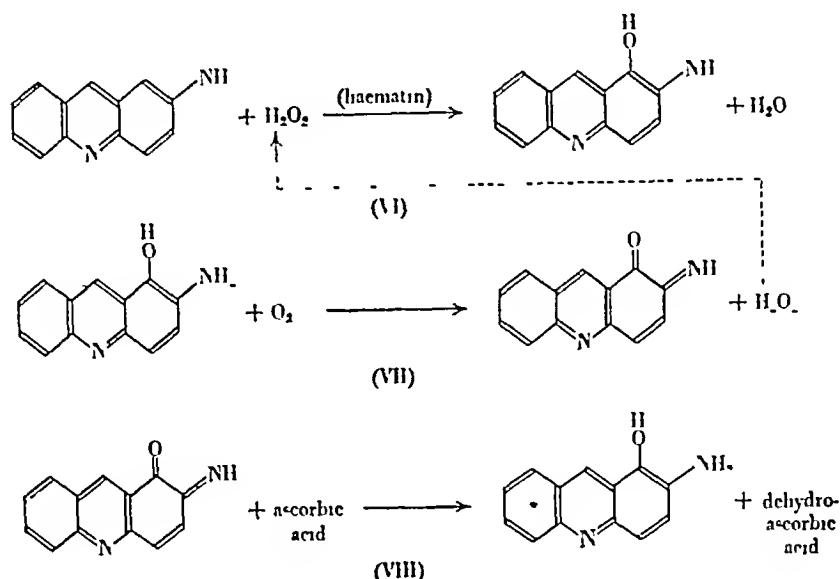
Table 6 *The effect with 2-aminoacridine at pH 8.5 and 25°*

(Each flask contained test substance, ascorbate and cytochrome *c* in the concentrations shown in Table 2. Borate buffer, pH 8.5 (0.035M) was used.)

	<i>t</i> (min)	<i>v</i> (μl O ₂ /min)	Percentage un-ionized at	
			pH 8.5	pH 7.0
2-Aminoacridine	120	0.8	78.0	10.3
2-Aminoacridine with H ₂ O ₂ , 10 ⁻³ M	0	5.0	—	—
3-Aminoacridine	60	1.6	99.8	94.1
3-Aminoacridine with H ₂ O ₂ , 10 ⁻³ M	0	10.5	—	—

Ames, Ziegenhagen & Elvehjem (1946), using 8-hydroxyquinoline as a copper-removing reagent in systems containing cytochrome oxidase, cytochrome c and ascorbic acid, reported a 'non-enzymic

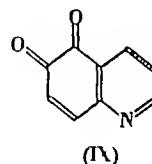
hydrogen peroxide only if 'substances capable of forming quinones are present', his examples of the latter being vanillin, *p*-phenylenediamine and fresh adrenal extract (but not adrenaline)



reaction in which a rapid oxygen uptake masked the enzyme reaction' This was without doubt due to the reaction of 8-hydroxyquinoline as described above (cf Table 5) A similar stimulation of oxygen uptake was found by Stotz, Harrer, Schultze & King (1937) when 8-hydroxyquinoline was added to washed liver brei to which ascorbate and haemolyzed blood had been added Washing the tissue removed blood pigments and other haematin compounds, and in the absence of haemolyzed blood no stimulation was found From the work of Lemberg, Legge & Lockwood (1939), the oxygen uptake on incubation of haemolyzed blood with ascorbate at pH 7 is known to be due to a coupled oxidation resulting in choleglobin formation, this process is stimulated by hydrogen peroxide and inhibited by catalase Stotz *et al* (1937) observed green pigment formation when 8 hydroxyquinoline was present, and it has since been found (Mr E C Foulkes, private communication) that choleglobin formation is markedly accelerated on addition of the carrier 7-amino-8-hydroxyquinoline (Table 5) to haemolyzed blood incubated with ascorbate at pH 7 and 37° The influence of 8-hydroxyquinoline may be explained by assuming preliminary peroxidative oxidation of the 8 hydroxyquinoline to a hydrogen carrier, which catalyzes the oxidation of the ascorbate with increased rate of oxygen uptake as described by Stotz *et al* The peroxide produced in this carrier-catalyzed oxidation should accelerate choleglobin formation, and the same explanation would apply to the experiment with pre-formed carrier

Tauber (1936) showed that horseradish peroxidase catalyzes the oxidation of ascorbic acid by

The possibility must be kept in mind that carriers of these types may also be formed *in vivo*, the introduction of hydroxyl groups into benzenoid rings being a well-known biological mechanism Specifically, Fuhner (1904) found that acridine fed to rabbits was excreted in the urine as 3,5 dihydroxy-acridine and later Fuhner (1906) showed that when quinoline was given to man a substance appeared in the urine which oxidized in air to the *o* quinone (IX)



Schonhofer (1942) found an interesting relationship among aminoquinoline compounds, in that gametocidal antimalarial activity was linked with the possibility of their biological conversion to quinonoid compounds

SUMMARY

1 Cytochrome c and methaemoglobin catalyze (*in vitro*) the oxidation, by hydrogen peroxide, of certain amino- and hydroxy acridines and -quinolines

2 The oxidized products are hydrogen carriers which efficiently promote the autoxidation of ascorbic acid or cysteine

3 The oxidized forms of the carriers appear, in the case of the amino compounds, to be *o*-quinonimines and, in the case of hydroxy compounds, *o*-quinones

4 The mechanism of the formation and action of the carriers is discussed

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Metabolism of Derivatives of Toluene

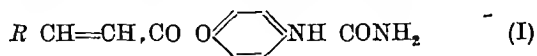
2 NUCLEAR METHYL-SUBSTITUTED DERIVATIVES OF N-PHENYLUREA

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The compounds included in this study were *N*-phenylurea and *N*-(*o*-, *m*- and *p*-tolyl)ureas. The metabolism of these substances does not appear to have been investigated previously, although two drugs which may be regarded as derivatives of *N*-phenylurea have received attention. 'Elbon' (I, $R = C_6H_5$) was shown by Mornaka (1922) to be



hydrolyzed in the rabbit to give benzoic acid and *N*-(*p*-hydroxyphenyl)urea, the former being excreted as hippuric acid and the latter as ether glucuronide, which was obtained as a crystalline potassium salt. Tsunoo (1935) showed that 'Ethynal', the corresponding 2-furyl derivative (I, $R = C_4H_3O$) gave rise in the dog to 2-(2'-furyl)-acrylic acid (excreted as such, and as its glycine conjugate) and the glucuronide of *N*-(*p*-hydroxyphenyl)urea, in the rabbit its fate was believed to be similar, although only the glucuronide was isolated. In man the only metabolite identified was furan-2-carboxylic acid (α -furoic acid).

There appears to be no account in the literature of the behaviour *in vivo* of the ureido group ($-NH-CO-NH_2$) attached to an aromatic ring, except for the two papers mentioned above, although compounds containing it were often claimed as metabolites of amino compounds by earlier workers. Such 'uramino' compounds include those stated to be derived from sulphanilic acid (Ville, 1892), anthranilic acid (Mitsuba & Ichihara, 1927), *m*-aminobenzoic acid (Salkowski, 1882) and various aminohydroxybenzoic acids (Pruszyński, 1893). All these compounds were believed to have been formed by the conjugation of urea with the amino group of the substance administered, ammonia being eliminated. These observations have, however, not been repeated in more recent investigations and it now appears likely that the compounds were artefacts, formed during isolation, which in most early investigations, involved evaporation of the urine to dryness (cf. Schmidt & Allen (1920) on Salkowski's (1873) uramino compound from taurine, Friedmann (1931) on excretion products of α -naphthylalanine and Bray, Lake, Neale, Thorpe & Wood

(1948b) on aminobenzoic acids) This process is now rarely used, having been superseded by direct extraction of the urine with ether or other solvents in continuous extractors

It has been shown that neither acetylation nor alkylation of the amino group of aniline alters the position in which the hydroxyl group enters as a result of biological hydroxylation aniline and acetanilide both give the para hydroxy derivative and acetanilide also gives the ortho compound (Schmiedeberg, 1878, Muller, 1887, Jaffé & Hilbert, 1888, Morner, 1889), *NN*-dimethyl- and *NN*-diethyl anilines both give para hydroxy derivatives in the rabbit (Horn, 1936, 1937) It was, therefore, of interest to determine whether modification of the amino group as it occurs in *N* phenylurea derivatives has any influence different from acetylation and alkylation

The metabolites which might be expected from phenylurea and the tolylureas would be the free amino compounds or their acetyl derivatives, hydroxylated derivatives either free or conjugated with glucuronic or sulphuric acid, and, in the case of the tolylureas, the corresponding benzoic acids formed owing to the oxidation of the methyl group These acids might be free or conjugated with glucuronic acid or glycine

The investigation carried out was similar to that described in Part 1 of this series (Bray & Thorpe, 1948), including a quantitative study of the effects of the four compounds upon the excretion of ether-soluble acid, diazotizable material, glucuronides and ethereal sulphates by the rabbit Qualitative studies included the isolation of oxidation and hydroxylation products similar in nature to those obtained from the acetotoluidides

MATERIALS AND METHODS

Materials The *N* phenylurea derivatives necessary for this investigation were readily prepared by the interaction of aqueous solutions of NaCNO and of the hydrochloride of the corresponding amino compound It was found that at least three recrystallizations from aqueous ethanol were necessary to give a product which gave a negligible colour on diazotizing and coupling Commercial preparations (*N* (*o* and *m* tolyl)urea (Genatosan) and *N*-phenylurea (Light and Co)) required similar treatment The two *N* (hydroxymethylphenyl)ureas were prepared from the corresponding aminocresols (Bray & Thorpe, 1948) and do not appear to have been described previously *N* (4-hydroxy 2 methylphenyl)urea formed large colourless prisms, *m* p 232°, which gave a transient weak blue colour with FeCl₃, the diazo reaction was negative, although a faint blue colour developed after 30 min The compound gave no indophenol reaction (Found N, 16.9 C₈H₁₀N₂O₂ requires N, 16.89%) *N* (4-hydroxy 3 methylphenyl)urea formed white needles, *m* p 203° It gave a transient faint brown coloration with FeCl₃, diazo and indophenol reactions were negative (Found N, 16.9 C₈H₁₀N₂O₂ requires N, 16.89%)

It was found that most phenylurea derivatives on keeping, either as solid or in solution, were hydrolyzed so that diazotizable material could be detected Table 1 shows results obtained with *N*-phenylurea The extent of this hydrolysis was somewhat greater in the case of *m* and *p* urodo benzoic acids and somewhat less with the three tolylureas It was for this reason that the compounds administered to the rabbits were recrystallized from aqueous ethanol immediately before an experiment In some cases several recrystallizations failed to give a product entirely free from diazotizable material Control experiments in which phenylurea derivatives were left standing in normal rabbit urine for 20 hr gave results for free amino compound (by diazo reaction) corresponding to about 2% It is, therefore, concluded that the free amino values observed in the experimental urines (Tables 2-5) are probably due to spontaneous hydrolysis rather than to any metabolic changes

Table 1 *Spontaneous decomposition of N-phenylurea at 20°*

Time (hr)	Percentage conversion to aniline	
	Aqueous solution (219 mg/100 ml)	Solid recrystallized from aqueous ethanol
0	—	0
0.5	0.5	—
2	0.5	0.4
10	1.9	0.8
98	3.2	3.1
196	7.2	7.7

The two *N* (hydroxymethylphenyl)ureas are more stable in the solid state When heated in solution some decomposition occurs, especially with *N* (4-hydroxy 3 methylphenyl) urea, which could only with difficulty be obtained with the ureido group intact by mild hydrolysis of its glucuronide (see p 140)

Diet and dosage The rabbits used were does of 2-3 kg The diet of rabbit pellets (Bruce & Parkes, 1946) and water previously described (Bray, Ryman & Thorpe, 1947) was maintained throughout the study The compounds investigated were administered by stomach tube as suspensions in water None of the tolylureas showed any toxic effect at a dose level of 0.25-0.30 g/kg, but phenylurea was appreciably toxic at this level, the rabbits recovering completely in a few hours

Methods of analysis These were essentially the same as those used in the acetotoluidide study (Bray & Thorpe, 1948), but included also estimation of the reducing value of urine hydrolyzed for 1.5 hr with HCl (cf Bray, Neale & Thorpe, 1946) The hydroxylation products formed as metabolites of phenylurea and *o* tolylurea did not reduce the Shaffer Hartmann reagent to any appreciable extent either before or after hydrolysis under the conditions used The hydroxylation product of *m* tolylurea was, however, slightly reducing after hydrolysis (3.44 mg ≡ 1 mg glucose) and allowance for this was made in the same way as for hydroxysulphapyridine (Bray *et al* 1946) Estimation of diazotizable material in hydrolyzed urine was not carried out owing to the difficulty of finding conditions which would give quantitative results The ureido compounds are not completely hydrolyzed under Bratton & Marshall's (1939) conditions even a tenfold increase in concentration

of acid failed to achieve complete hydrolysis (This is surprising in view of the spontaneous hydrolysis of these compounds already referred to) The use of this more concentrated acid is attended by the difficulty of adjusting the acidity to that suitable for development of the diazo colour which is retarded or inhibited by excess of acid Another source of error is that, unless suitable precautions are taken, aniline and toluidine may be lost in steam during hydrolysis, even from acid solution Finally, there is the complication of the choice of a suitable standard since hydrolysis of the urines may yield more than one diazotizable compound, and the diazo colours given by the compounds differ considerably, e.g. aniline and *p* aminophenol, toluidine, amino cresol and aminobenzoic acid The standards used for the diazo estimations, which were performed, were aniline sulphate for phenylurea, *o* toluidine hydrochloride for *o* tolylurea and *m* and *p* aminobenzoic acids for the corresponding tolylureas An attempt was made to devise a method capable of distinguishing between acetamido and ureido compounds, but it was found that even under the mildest conditions necessary for the hydrolysis of the former, the ureido derivatives were hydrolyzed to some extent

RESULTS

Quantitative studies

Excretion of normal metabolites The average daily excretion of normal metabolites by individual rabbits used in this study was as follows ether soluble acid (as hippuric acid), 634–853 mg, the day to day variation for each rabbit usually being less than $\pm 7\%$ of the mean, reducing material (as glucuronic acid), 110–202 mg, the day to day variation for each rabbit usually being less than $\pm 13\%$ of the mean, average increase of reducing material on hydrolysis, 76%, ethereal sulphate, 32–63 mg SO_3 , the day to day variation for each rabbit usually being within $\pm 10\%$ (i.e. approx 5 mg SO_3) of the mean

Metabolism of *N* phenylurea Table 2 summarizes the results obtained It can be seen that an average of 46% of the dose is hydroxylated and conjugated with sulphuric acid (20%) and glucuronic acid (26%) The principal hydroxylation product is *N* (*p* hydroxyphenyl)urea and some, at least, is excreted unconjugated, as evidenced by the purple colour which phenylurea urines give with FeCl_3 The remainder of the dose is presumably excreted unchanged, but it was not possible to demonstrate this quantitatively, owing to the stability of the ureido group

Metabolism of *o* tolylurea The quantitative results obtained are given in Table 3 Little or no oxidation of the methyl group occurs, since there is no increase in excretion of ether soluble acid or ester glucuronide, but an average of 35% of the dose is hydroxylated, about 4% of the resulting *N*-(4-hydroxy 2 methylphenyl)urea being conjugated with sulphuric acid and 31% with glucuronic acid

Metabolism of *m* tolylurea As may be seen from Table 4, which contains the quantitative results obtained, an average of 45% of the dose is oxidized to a carboxylic acid, of which the main part (41%) is excreted unconjugated and only 4% as ester glucuronide In addition hydroxylation takes place (14%), the product being excreted conjugated as both ethereal sulphate (10%) and ether glucuronide (14%) The reducing properties of the hydroxylation product (*N* (4-hydroxy 3 methylphenyl)urea) under the conditions of the estimation were allowed for as previously described

Metabolism of *p* tolylurea Table 5 summarizes the results obtained About 80% of the dose is oxidized to *p* ureido benzoic acid, of which only a small amount (6%) is excreted in conjugated form

Stability of ureido compounds to rabbit liver extracts The experiments described above do not preclude the possibility that slight hydrolysis of the ureido group occurs *in vivo* If this does occur it might be expected that extracts of rabbit liver would bring about the hydrolysis *in vitro* It was, however, found that incubation of *N* phenylurea, *N* (*p* hydroxyphenyl)urea, the three tolylureas and *m* and *p* ureidobenzoic acids (c. 0.01M in buffer pH 7.4) with rabbit-liver extract prepared as previously described (Bray, James, Ryman & Thorpe, 1948a) gave rise to no liberation of diazotizable material beyond that formed by spontaneous hydrolysis in the controls Similarly, *N* phenylurea was not hydrolyzed by rabbit kidney extract

Qualitative studies

***N*-Phenylurea** The urine excreted by rabbits which had received phenylurea gave a purple colour with FeCl_3 , suggesting that an unconjugated phenolic compound was excreted We were not able to isolate the compound responsible, however, by means of ether extraction at various values of pH The quantitative results given in Table 2 suggest that about half the dose might be excreted unchanged, but it was not possible to isolate *N* phenylurea from the urine by extraction with either ether or methyl ethyl ketone (The latter extracts *N* phenylurea which has been added to urine) A small amount of phenylurea was obtained during the isolation of the glucuronide (*qv*), but

Table 2 Metabolism of *N* phenylurea in the rabbit

Rabbit no	Dose (g/kg)	Percentage of dose excreted as		
		Ether glucuronide	Free amino compounds	Ethereal sulphate
152	0.3	21	0.7	25
		17	<1.0	24
		24	0	—
156	0.3	31	2.4	16
		35	<1.0	—
		33	0	—
160	0.3	25	0	15
		12	<1.0	—
		32	0	—
	Av.	26	<1.0	20

it was not possible to obtain an amount approximating to that unaccounted for

Phenylurea urine was hydrolyzed with acid (cf Bray & Thorpe, 1948), neutralized and continuously extracted with ether. From 9.0 g *N*-phenylurea given to ten rabbits 0.6 g of a crystalline compound, m.p. 186°, was obtained. It gave a positive diazo reaction and a purple brown colour with FeCl₃. Mixed with an authentic specimen of *p*-aminophenol (m.p. 187°) it melted at 187°. The identity of the compound was further confirmed by comparing its *N*-acetyl derivative (m.p. 168°) with authentic *p*-acetamidophenol (m.p. 169°). The mixed m.p. was 169°. In the diazo tests on the crude extract a yellow colour was obtained with HNO₂, it was thought that this might be due to the presence of an *o*-aminophenol derivative, but attempts at its isolation were unsuccessful.

From 6 g phenylurea given to six rabbits a glucuronide was isolated as previously described (Bray *et al.* 1947) from which a crystalline potassium salt (150 mg) was obtained, as described by Morinaka (1922) and Tsunoo (1935). The properties are given and compared in Table 6 with those of the compounds obtained by the Japanese workers. We were unable to isolate *N*-(*p*-hydroxyphenyl)urea by the hydrolysis of the glucuronide, but on sublimation of the potassium salt with soda lime, *p*-aminophenol was obtained and identified as previously described. The glucuronide did not form a crystalline barium salt. During an attempt to isolate the glucuronide through its barium salt a small amount of *N*-phenylurea was isolated.

o-Tolylurea. Extraction of neutral *o*-tolylurea urine with ether gave a small quantity of crystals, m.p. 190°, which were identified as *o*-tolylurea. Yield, 8% of the dose. The

Table 3 *Metabolism of o-tolylurea in the rabbit*

Rabbit no	Dose (g /kg)	Percentage of dose excreted as				
		Ether soluble acid	Ester glucuronide	Ether glucuronide	Free amino compounds	Ethereal sulphate
152	0.3	0	0	28	<1.0	6
		0	0	10	—	—
		0	0	28	1.4	—
		0	0	38	<1.0	—
156	0.3	0	0	33	<1.0	—
		15	0	17	—	0
		0	0	37	1.8	—
		0	0	32	<1.0	—
166	0.3	0	0	28	<1.0	—
		0	0	19	—	—
		0	0	44	<1.0	5
		0	0	30	<1.0	—
168	0.3	12	0	56	<1.0	—
		27	0	20	—	—
		0	0	31	1.7	3
		8	0	37	<1.0	—
	Av	4*	0	31	<1.0	4

* Since there is no other evidence of oxidation of the methyl group it seems likely that the positive values in this column are due to abnormal fluctuations in the base line values, and that the true figure here is 0.

Table 4 *Metabolism of m-tolylurea in the rabbit*

Rabbit no	Dose (g /kg)	Percentage of dose excreted as								
		Ether soluble acid	Ester glucuronide	Ether glucuronide	Free ammo compounds	Ethereal sulphate				
178	0.3	44	7	11	1.4	12				
		46	4	12	1.8	—				
		27	0	13	1.1	—				
		40	3	13	—	—				
182	0.3	42	7	12	1.3	—				
		31	0	18	2.6	8				
		36	6	9	1.6	—				
		36	4	21	—	—				
190	0.3	46	4	15	1.5	—				
		41	3	17	2.2	—				
		49	4	8	1.5	—				
		40	3	19	—	11				
191	0.3	52	9	14	1.4	—				
		48	5	14	1.8	—				
		36	4	6	1.5	—				
		41	5	20	—	—				
		Av	41	4	14	1.5	9			
		</								

Table 5 *Metabolism of p-tolylurea in the rabbit*

Rabbit no	Dose (g/kg)	Percentage of dose excreted as				
		Ether soluble acid	Ester glucuronide	Ether glucuronide	Free amino compounds	Ethereal sulphate
152	0.3	52	2	0	2.3	0
		81	4	0	1.7	—
		65	4	0	2.2	—
		88	—	—	—	—
156	0.3	85	5	0	2.1	1
		78	8	0	2.8	—
		79	8	0	2.1	—
		85	—	—	—	—
160	0.3	—	8	0	2.9	—
		—	7	0	1.7	—
		—	5	0	1.8	0
168	0.3	96	—	—	—	—
		80	—	—	—	—
		87	—	—	—	—
		Av	80	6	2.2	0

Table 6 *Properties of potassium salts of 4-ureidophenylglucuronide*

	From <i>N</i> -phenylurea in rabbit	From 'Elbon'* in rabbit	From 'Ethynal'†		Calc for $C_{12}H_{16}N_2O_8K$
			In dog	In rabbit	
% N	7.69	7.59	7.5	7.31	7.65
% K	10.70	10.62	11.07	—	10.68
$[\alpha]_D$ in water (°)	-70.1 at 23	-74.99 at 20	-67.8 at 29	-59.3 at 23	—
MLP (decomp) (°)	257	231	243	231	—

* Morinaka (1922)

† Tsunoo (1935)

urine was acidified and re extracted, but only hippuric acid was isolated. No acetotoluidide was detected.

Extraction of neutral, hydrolyzed urine gave a crystalline product which darkened rapidly. Treatment with charcoal and recrystallization from aqueous ethanol containing SO_2 gave 50 mg light yellow crystals, m.p. 178°, which gave a red-purple coloration with $FeCl_3$ and positive diazo and indophenol reactions mixed with an authentic specimen of 2-amino-5-hydroxytoluene (m.p. 179°), m.p. 179°. The acetate of the compound isolated (m.p. 125°) gave a blue-violet colour with $FeCl_3$ and was shown to be identical with an authentic specimen of 2-acetamido-5-hydroxytoluene.

Crystalline barium or potassium salts of the glucuronide could not be obtained and attempts to obtain a crystalline glucuronide were unsuccessful, the product being a syrup. On hydrolysis of this with 2N HCl 50 mg of crystals, m.p. 230°, were obtained. The identity of this compound with *N*-(4-hydroxy-2-methylphenyl)urea was shown by comparison with an authentic specimen (Found N, 16.6. Calc for $C_8H_{10}N_2O_2$ N, 16.89%).

m-Tolylurea. Ether extraction of *m*-tolylurea urine as collected yielded no product, but from the acidified (pH 1.5) urine a compound, m.p. 290° (decomp), was obtained in a yield corresponding to 25% of the dose administered. The compound had the same properties as synthetic *m*-ureidobenzoic acid and contained 15.6% N, calc for $C_8H_8N_2O_3$ N, 15.56%. After hydrolysis for 2 hr with 2N-HCl, extraction with ether gave a crystalline product, m.p. 177°, which was shown to be identical with authentic *m*-aminobenzoic acid, and its identity was further confirmed by comparison of the *N*-acetyl derivatives. It is, therefore, concluded that the compound originally isolated

was *m*-ureidobenzoic acid. No *m*-acetotoluidide or toluidine was isolated.

Ether extraction of neutral, hydrolyzed *m*-tolylurea urine yielded yellow-brown crystals, m.p. 174°, which gave with $FeCl_3$ a red brown colour, changing slowly to red purple, and a positive indophenol reaction. On diazotizing and coupling with *N*-(1-naphthyl)ethylenediamine dihydrochloride a pale blue colour slowly giving place to purple was observed, which is characteristic of some amino cresols, e.g. 5-amino-2-hydroxytoluene, 2-amino-5-hydroxytoluene. Yield, 40 mg from four doses of 0.9 g. This compound was identical with an authentic specimen of 5-amino-2-hydroxytoluene (m.p. 175°), mixed m.p. 174°. Its acetyl derivative melted at 180°, and did not depress the melting point of a synthetic specimen of 5-acetamido-2-hydroxytoluene. This does not give a colour with $FeCl_3$. (Found N, 8.31. Calc for $C_9H_{11}NO_2$ N, 8.48%.)

Attempts to obtain a crystalline glucuronide (or salt) were unsuccessful, only a syrup being obtained. The syrup was hydrolyzed by refluxing for 2 hr with 2N HCl and extracted with ether. The ether soluble material was divided into two parts. From one 5-amino-2-hydroxytoluene was obtained and identified as above. The other part was acetylated, giving 5-acetamido-2-hydroxytoluene and *m*-acetamidobenzoic acid. This finding is in agreement with the results shown in Table 4, indicating the presence of both ester and ether type glucuronides. After several attempts to obtain *N*-(4-hydroxy-3-methylphenyl)urea by milder hydrolysis of the glucuronide syrup (20 min on boiling water bath with N HCl), about 5 mg straw coloured needles, m.p. 195°, were obtained in one experiment. These gave a brown colour with $FeCl_3$ and, after hydrolysis, indophenol

and diazo reactions similar to those given by 5 amino-2-hydroxytoluene. Mixed m.p. with authentic *N*-(4-hydroxy-3-methylphenyl)urea (m.p. 198°), 195°. The amount was insufficient for purification for analysis. Small amounts of *m*-tolylurea were also obtained.

p-Tolylurea. Extraction of acidified *p*-tolylurea urine yielded an acid which did not melt below 300°. Yield 1.2 g. from a total dose of 3.6 g. After recrystallization from aqueous ethanol it was found to contain 16.0% N (Calc. for $C_8H_9N_2O_3$, N, 15.56%). Acid hydrolysis gave a crystalline product, m.p. 187°, which was shown to be identical with *p*-aminobenzoic acid (m.p. 189°), mixed m.p. 189°. This was confirmed by comparison of the acetamido compounds. It is, therefore, concluded that the product isolated from urine was *p*-ureidobenzoic acid. No other metabolite was isolated.

DISCUSSION

Table 7 summarizes the quantitative results obtained in this investigation, together with the corresponding results obtained with acetotoluides (Bray & Thorpe, 1948). It can be seen that the metabolic fates of the two types of compound are very similar, the main differences being quantitative rather than qualitative, e.g. the percentage conjugation of the hydroxylation products with sulphuric and glucuronic acids.

Although the ureido group in the compounds studied appears to be stable *in vivo*, as evidenced by the fact that various metabolites were isolated with it intact, the tendency to spontaneous decomposition suggests that some hydrolysis might be expected to occur. It is theoretically possible that amino compounds arising in this way may be acetylated before excretion. Attempts were made to identify acetamidobenzoic acids and acetotoluides among the excretion products, in every case without success. Owing to the difficulties of distinguishing between and separating *N*-phenylurea derivatives and acetamido compounds, we do not regard the question as having been finally settled, although enzyme

experiments lend support to the view that the ureido group is stable *in vivo*.

Other compounds in which a ureido group is attached directly to the aromatic ring do not appear to have been investigated, but the literature provides some information as to the stability of this group attached to aliphatic or other residues. Koehne (1895) stated that biuret was excreted unchanged by the dog, and Gaebler & Keltch (1926) found over 50% of hydantoin or hydantoic acid was excreted by the dog as hydantoic acid, there being no evidence of further breakdown. Several diphenylhydantoins, however, are excreted by man and dog as the diphenylhydantoic acids, and, to a much greater extent, as the corresponding α -aminodiphenylacetic acids (Hine & Kozelka, 1943). Benzoylurea (Koehne, 1895) and benzylidenedureide (Bulow, 1894) in the dog appear to lose the ureido group intact with the formation of urea.

The hydroxylation products of the tolylureas resemble exactly those from the acetotoluides, all their constitutions being in agreement with the general rule suggested previously (Bray *et al.* 1948*b*). It is clear, therefore, that modification of the aromatic amino group by conversion to a ureido group does not change the nature of its influence on the position taken up by the entering hydroxyl group.

SUMMARY

1 The metabolism of the three *N*-tolylureas in the rabbit has been shown to be similar to that of the corresponding acetotoluides.

2 The ureido group in these compounds appears to be stable in the rabbit and to rabbit-liver extracts *in vitro*.

3 *o*-Tolylurea is converted to *N*-(4-hydroxy-2-methylphenyl)urea (35% of the dose) which is excreted as ethereal sulphate (4%) and glucuronide (31%). The methyl group is not oxidized to any

Table 7 Comparison of metabolism of *N*-tolylureas, acetotoluides and *N*-phenylurea in the rabbit

Compound	Percentage of compound						
	Excreted as ether soluble acid	Excreted as ester glucuronide	Oxidized to carboxylic acid	Excreted as ether glucuronide	Excreted as ethereal sulphate	Hydroxylated	Excreted with free amino group§
<i>o</i> -Tolylurea	0	0	0	31	4	35	<1.0
<i>o</i> -Acetotoluidide*	8	—	6†	—	32	32‡	5.6
<i>m</i> -Tolylurea	41	4	45	14	10	24	1.5
<i>m</i> -Acetotoluidide*	34	—	25†	—	10	10‡	Trace
<i>N</i> - <i>p</i> -Tolylurea	80	6	86	0	0	0	2.2
<i>p</i> -Acetotoluidide*	78	10	96†	—	0	0‡	0.8
<i>N</i> -Phenylurea	—	0	—	26	20	46	<1.0

* Bray & Thorpe (1948).

† From total diazotizable material.

‡ May be low since no ether glucuronide value is included.

§ All the values recorded in this column, with the exception of that for *o*-acetotoluidide, are probably due to spontaneous hydrolysis. The acetotoluides hydrolyze spontaneously, although to a smaller extent than does *N*-phenylurea (Bray, Lake & Thorpe, unpublished).

appreciable extent Some *o*-tolylurea is excreted unchanged

4 *m*-Tolylurea is oxidized to *m*-ureidobenzoic acid (45 %), 4 % of which is excreted as ester glucuronide, and hydroxylated to *N* (4 hydroxy-3 methylphenyl)urea (24 %), of which 10 % is excreted as ethereal sulphate and 14 % as ether glucuronide Some *m*-tolylurea is excreted unchanged

5 *p*-Tolylurea is almost completely oxidized to *p*-ureidobenzoic acid (86 %), of which only 6 % is conjugated as ester glucuronide, the remainder being excreted free

6 *N*-Phenylurea is partly hydroxylated to *N* (*p* hydroxyphenyl)urea (46 %) which is conjugated almost equally with sulphuric acid (20 %) and glucuronic acid (26 %) Some *N* phenylurea is excreted unchanged 4-Ureidophenylglucuronide was isolated from *N*-phenylurea urine as its crystalline potassium salt

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The Effect of Deamination and Esterification on the Reactivity of Collagen

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The most important reactive groups in collagen are amino, guanidino and carboxyl, all of which are present in comparatively large numbers, few phenolic, hydroxyl and imidazole groups are present and indole groups are absent (Bowes & Kenten, 1948a) A study has now been made of the effect of modification of the amino, guanidino and carboxyl groups on the acid and base binding capacity, and on the swelling of collagen The combination of the modified collagens with vegetable tannins, basic chromium salts and formaldehyde has also been investigated primarily because of the importance of these three classes of compound in the conversion of collagen to leather Moreover, the fixation of metals

by proteins is of general interest, and information concerning the factors affecting the fixation of tannins may help to throw light on the association of tannins and proteins in nature

The amino groups of proteins can readily be converted to hydroxyl groups by the action of nitrous acid The reaction is not entirely specific, however, since at low pH values, or if the reaction is prolonged, modification of the guanidino, imidazole, tyrosine and glutamide groups may also take place (for review of literature see Olcott & Fraenkel Conrat, 1947) With soluble proteins, reaction with the amino groups is rapid and other reactions are probably negligible, but with fibrous proteins, such

as collagen, the reaction must be prolonged to allow time for the nitrous acid to diffuse into the interior of the fibres, and some modification of other groups probably occurs. Previous workers who have used this method with collagen did not determine the extent of specificity of the reaction (Thomas & Foster, 1926, Thomas & Kelly, 1926, Gustavson, 1926, Meunier & Schweikert, 1935, Atkin, 1937, Bowes & Pleass, 1939, Highberger & Retzsch, 1939, Wilson & Yu, 1941, Gustavson, 1943, Lollar, 1943, Chang, Yen & Chen, 1944, Theis, 1945).

Modification of the guanidino groups has received little attention and no satisfactory method appears to be available. Treatment with hypochlorite, as in the Sakaguchi reaction, has been found to destroy all the arginine present in soluble proteins, but histidine, tyrosine, tryptophan and lysine are also affected, though to a lesser extent (Sakaguchi, 1925). Only about 43% of the arginine in collagen is affected by the reaction (Highberger & Salcedo, 1940). In the present investigation, it was again found that only 40–50% of the arginine in collagen was destroyed by this treatment. Roche & Morgue (1946) have shown that the reactivity, in the Sakaguchi reaction, of the guanidino group in amino acid derivatives is influenced by the presence of other groups, some of which, notably aliphatic hydroxyl, prevent over 90% of the guanidino groups from reacting. It is possible that the difficulty of removing more than about half the guanidino groups from the collagen is related to the same cause. Since the reaction was obviously not specific to the guanidino groups, and was accompanied by extensive general breakdown of the collagen, it was not studied in detail.

A number of the reagents which react with the carboxyl groups of proteins, e.g. diazomethane, acetic anhydride, keten, methanol, are not specific to carboxyl groups (Herriott, 1947, Olcott & Fraenkel Conrat, 1947), and with some the conditions necessary for reaction cause breakdown of the protein structure (Fodor & Epstein, 1937). Blackburn, Carter & Phillips (1941) and Blackburn & Phillips (1944) have studied the methylation of wool with methyl sulphate and methyl halides, and conclude that the main reaction is esterification of the carboxyl groups. It appeared, therefore, that these methods were likely to be satisfactory for modifying the carboxyl groups of collagen without affecting other groups to any appreciable extent, and with minimum risk of degradation.

EXPERIMENTAL METHODS

Material

The collagen was prepared from sheepskins which had been dewooled with $\text{Ca}(\text{OH})_2$ and Na_2S ('limed'), treated with pancreatic trypsin ('bated'), and preserved with H_2SO_4

and NaCl ('pickled'). The skins were washed free of acid and salt, dehydrated with acetone, cut into strips 1×5 cm (the edges of the skins being discarded), brought to equilibrium with distilled water (pH c 5), and finally dehydrated with acetone. The titration curves of three batches of collagen prepared as above (*A*, *B* and *C*) are given in a previous paper (Bowes & Kenten, 1948*b*), they are referred to in text and tables as collagen *A*, *B* and *C*, with the prefixes *D* and *M* to denote deaminated and methylated, respectively.

Treatment of collagen

Deamination. The method used was based on that of Thomas & Foster (1926). Collagen (100 g) was soaked in distilled water overnight, 100 g NaNO_2 then added, followed by 100 ml glacial acetic acid. A stream of CO_2 was passed through the solution to minimize oxidation of nitrous to nitric acid and to agitate the solution. After 24 hr, a further 100 g NaNO_2 and 100 ml acetic acid were added and the reaction allowed to proceed for another 24 hr. The collagen was rinsed with water, washed to remove acid in several changes of water containing 10% (w/v) NaCl to minimize swelling, brought to equilibrium with distilled water (pH 5.0), and dehydrated with acetone. Two batches of collagen *C* (*DC*₁ and *DC*₂) and one of collagen *A* (*DA*) were treated in this way.

Methylation. Methods used were similar to those of Blackburn *et al* (1941). (1) Dimethyl sulphate. Collagen (50 g) was shaken with 500 ml *M* Na acetate buffer (pH 7.5) and 25 ml methyl sulphate for 1 hr. This procedure was repeated fourteen or more times. To reduce the extreme swelling of the methylated collagen it was placed in a 10% (w/v) solution of NaCl for 20 hr, then washed in distilled water until free from Cl^- , and dehydrated with acetone. (2) Methyl bromide. The collagen was brought to equilibrium with a borate buffer at pH 9.0, dehydrated with acetone, air dried, and immersed in methyl bromide for 14 days. The latter was then allowed to evaporate, and the collagen washed as before and dehydrated with acetone. In some cases the collagen was rebuffered and treated for a further 14 days with methyl bromide. Several batches of collagen *A* (*MA*₁ to *MA*₄) and of collagen *C* (*MC*₁ to *MC*₆) were methylated, also the oxhide collagen prepared as previously described (Bowes & Kenten, 1948*a*).

Combination of modified collagen with tannic acid, mimosa tannins, chromium and formaldehyde. The modified collagen (2 g) in the form of pieces 1 cm square, and a piece 1×5 cm for shrinkage temperature determinations were soaked in water overnight and then placed in 100 ml of the following tanning solutions adjusted to different pH values for the times stated. Approximate equilibrium should have been reached in these times.

- (1) 10% (w/v) tannic acid (British Drug Houses, Ltd) for 7 days
- (2) 10% (w/v) tannic acid + 0.5M NaCl for 7 days
- (3) 10% (w/v) commercial mimosa tannin extract for 7 days
- (4) 3.4% (w/v) chromic sulphate for 4 days
- (5) 0.36% (w/v) formaldehyde for 3 days

All pH values were attained with HCl or NaOH. The tannin solutions were initially adjusted to the required pH and then subsequently twice daily during the treatment. The pH of the $\text{Cr}_2(\text{SO}_4)_3$ solutions was adjusted after 24 hr, and maintained at this pH value by frequent further additions.

during the following 48 hr, no additions were made in the last 24 hr and the pH fell slightly during this time. The pH's of the formaldehyde solutions were initially adjusted, and then allowed to fall during the treatment.

On the completion of the treatment the pieces were drained, washed in Wilson Kern extractors (Wilson & Kern, 1921) with 9 l distilled water for 20 hr and air dried.

Analysis of products

Total N, amide N, amino N and arginine The methods have been described previously (Bowes & Kenten, 1948a, b).

Lysine This was determined using a specific decarboxylase (Gale, 1945). Though this method may also determine an unknown fraction of the hydroxylysine it gives an indication of the extent to which ϵ amino groups are modified. Recent evidence (Heathcote, 1948) suggests that the apparent reaction of hydroxylysine with lysine decarboxylase may in fact be due to contamination of the hydroxylysine with lysine.

O Methyl groups These were determined as methyl iodide using a semimicroalkoxyl apparatus (Clark, 1932, 1939, Viebock & Schwappach, 1930). Methionine also yields methyl iodide by this method (Baernstein, 1932, 1936). The oxhide and sheepskin collagens gave values of 0.08 and 0.05% of methyl, respectively.

N Methyl groups The method is described by Pregl (1930). *O* Methyl and *N* methyl contents are expressed as a percentage of methyl on moisture- and ash free collagen and are corrected for the methionine content of the collagen.

Chloride and sulphate The former was determined as described by Highberger & Moore (1929). In the determination of SO_4^{2-} , methylated collagen (3 g) was heated for 3 hr on a steam bath with 30 ml 2*N* HCl. The solution was evaporated to dryness, the residue dissolved in 100 ml hot water and boiled with charcoal (Norite) for 15 min. The solution was filtered, the charcoal extracted with boiling water, and the combined filtrates neutralized with 2 ml 5*N* HCl, and the SO_4^{2-} determined as BaSO_4 . Collagen gave no SO_4^{2-} by this method, and recovery tests showed an accuracy of $\pm 5\%$.

Tannin in combination with collagen This was determined by drying in a vacuum oven at 100–102° for 6 hr and weighing. The amount of tannin fixed/100 g moisture and ash free collagen was then calculated from the increase in weight of the collagen.

Chromium The method used is described by Davies & Innes (1944). Results are expressed as g Cr_2O_3 /100 ml and mmol. Cr/g moisture- and ash-free collagen.

Formaldehyde The treated collagen (2 g) was steam distilled with 40 ml 2*N* H_2SO_4 , 500 ml distillate being collected. The formaldehyde was precipitated from suitable samples of distillate with dimedone (Yoe & Reid, 1941).

Shrinkage temperature The temperature at which shrinkage occurs in water is a property characteristic of both collagen and treated collagens. It has been related to cross linking in the protein structure (Theis, 1946). A modified form of the apparatus described by Kuntzel (1943) was used, in which a stream of air was blown continuously round the side arm to aid circulation. When the shrinkage temperature exceeded 100° the tanned collagens were wetted by immersion in water under reduced pressure and the determination then made in liquid paraffin. The shrinkage tempera-

ture so determined is the same as that determined in water under pressure.

Titration and swelling curves These were determined as previously described (Bowes & Kenten, 1948a, b).

RESULTS

Except where otherwise stated, results are expressed on a moisture- and ash-free basis. Amino N contents, etc., are expressed as mmol/g rather than as a percentage of total N, since variations in the N content of the modified collagens render the latter method of expression unsuitable for comparative purposes.

Analysis

Deamination The deaminated collagens had the characteristic orange yellow colour associated with proteins which have been treated with nitrous acid (cf Philpot & Small, 1938). Reaction of the amino groups was almost complete in all the samples examined (Table 1). As with other proteins (Steudel & Schumann, 1929, Wiley & Lewis, 1930) there was some loss of arginine (c. 20%).

The amide N was higher than that of the original collagen, and this additional NH_2 may have come from $-\text{NH}-\text{CN}$ groups formed by the action of the nitrous acid on guanidino groups (see p 149). The increase in amide N was approximately equal to the decrease in arginine content.

Methylation The methylated collagens varied from brown to grey in colour and in two cases were almost black. They had a smell of methylamines. The methoxyl content of the different collagens treated with methyl sulphate varied from 0.94 to 1.57% (as methyl), the maximum methoxyl content being attained in 14 methylations (see oxhide collagen, Table 2). Variations in methoxyl content are probably due to slow hydrolysis of these groups during washing (see below). Methylation with methyl bromide introduced rather fewer methoxyl groups. Deamination of the collagen did not affect the extent of methylation with methyl sulphate, but increased the methoxyl groups introduced by methyl bromide to the same value as that obtained with methyl sulphate. This suggests that the lower values obtained with methyl bromide on untreated collagen are due, not to inability to introduce the same number of methoxyl groups as methyl sulphate, but to the difficulty of keeping the collagen at a suitable pH for the reaction to proceed to completion; diminution in the number of basic groups will reduce the change in pH of the collagen which occurs as a result of methylation of the carboxyl groups, and hence makes it easier to keep the collagen at the required pH.

Methylated collagen fixes SO_4^{2-} and Cl^- approximately equivalent to the methoxyl groups introduced. Blackburn *et al* (1941) found that when wool was methylated with

Table 1 *Analyses of collagens and deaminated collagens*

(Results expressed on moisture and ash free basis except where otherwise stated)

	Untreated <i>C</i>	Treated with HNO ₂		Untreated <i>A</i>	Treated with HNO ₂ <i>DA</i> ₁
		<i>DC</i> ₁	<i>DC</i> ₂		
Moisture (%) air dry	16.2	6.3*	16.0	18.1	14.1
Ash (%) air dry	0.23	1.78	0.23	0.14	0.58
Total N (%)	17.2	16.9	—	17.3	17.3
Amide N (mmol/g)	0.25	0.38	0.39	0.30	0.38
Amino N (mmol/g)	0.36	0.05	—	—	0.04
Lysine† (mmol/g)	0.30	—	Nil	0.31	0.01
Arginine (mmol/g)	0.11	—	0.35	0.43	0.37

* The low value is due to the collagen having been dried in a desiccator over anhydrous CaCl₂ for some days, other samples air dry

† May include some hydroxylysine

Table 2 *The methylation of collagen and deaminated collagen with methyl sulphate and with methyl bromide*

Sample	OCH ₃ groups introduced (as CH ₃)		Anions (in equiv/g collagen)		
	(%)	(mmol/g)	Chloride*	Sulphate	Total
Methylated with methyl sulphate					
Oxide collagen (free carboxyl groups, 0.87 mmol/g)					
Methylated { <i>MN</i> ₁	1.19	0.79	Nil	0.64	0.64
14 times { <i>MN</i> ₂	0.94	0.63	Nil	0.52	0.52
Methylated { <i>MN</i> ₃	1.37	0.91	Nil	0.78	0.78
16 times { <i>MN</i> ₄	1.22	0.81	Nil	0.70	0.70
Methylated { <i>MN</i> ₅	1.15	0.77	Nil	0.65	0.65
18 times { <i>MN</i> ₆	1.24	0.83	Nil	0.75	0.75
Collagen <i>A</i> (free carboxyl groups 0.96 mmol/g)					
<i>MA</i> ₁	1.25	0.83	0.61	—	—
<i>MA</i> ₂	1.32	0.88	0.73	0.07	0.80
<i>MA</i> ₃	1.40	0.99	0.58	0.40	0.98
<i>MA</i> ₄	1.32	0.88	0.50	0.34	0.84
Collagen <i>C</i> (free carboxyl groups, 1.01 mmol/g)					
<i>MC</i> ₁	1.32	0.88	—	—	—
<i>MC</i> ₂	1.57	1.05	0.52	0.52	1.04
<i>MC</i> ₃	1.18	0.79	0.65	—	—
<i>MC</i> ₄	1.04	0.60	—	—	—
Collagen <i>A</i> after treatment with nitrous acid					
<i>MDA</i> ₁	1.32	0.88	0.42	0.05	0.47
Collagen <i>C</i>					
Methylated with methyl bromide					
Methylated 14 days <i>MC</i> ₅	0.35	0.23	0.11	—	0.11
Methylated 28 days <i>MC</i> ₆	0.85	0.57	0.48	—	0.48
Collagen <i>C</i> after treatment with nitrous acid					
Methylated 28 days <i>MDC</i> ₁	1.22	0.82	0.30	—	0.30

* Arises from displacement of SO₄²⁻ by Cl⁻ during immersion of methylated collagen in NaCl

methyl iodide and bromide, I⁻ and Br⁻ were held in excess of the methoxyl groups, and attributed this to *N* methylation. Attempts to determine the percentage of *N* methyl groups were unsuccessful. Under the conditions of Pregl's method proteins and amino acids yield appreciable amounts of volatile iodides, and large and variable values for the apparent *N* methyl are obtained (Burns, 1914, Geake & Nierenstein, 1914, Lindley & Phillips, 1947). Determination of *N* methyl groups in methylated collagen gave values of the same order as those obtained with the original collagen. Though it may be unwise to conclude that no *N* methylation takes place, it appears that it only occurs to a small extent, if at all. The amino N of the collagen methylated with methyl bromide (*MC*₆) and with methyl sulphate

(*MC*₄) was 0.24 and 0.31 mmol/g, respectively, compared with a value of 0.36 mmol/g for the original collagen. The amino N of collagen *MA*₄ methylated with methyl sulphate was 0.30 mmol/g.

Stability Methoxyl groups were only slowly split by hydrolysis when the methylated collagens were immersed in buffer solutions between pH 2 and 8, but outside this range the stability decreased rapidly with rise or fall in pH (see Fig. 1). A similar stability curve for methylated wool constructed from the data of Blackburn *et al.* (1941) is given for comparison. Soaking in water at pH 5.0 for 20 days

reduced the methoxyl content from 1.38 to 1.12% (as methyl)

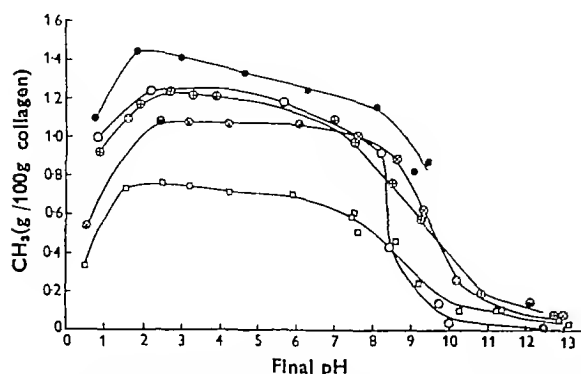


Fig 1 Stability of OCH_3 groups introduced into collagen, deaminated collagen and wool in pH range 0.5–13.0. Percentage CH_3 after soaking in universal buffer solution for 2.5 days: \bigcirc — \bigcirc , collagen esterified with methyl sulphate, \square — \square , collagen esterified with methyl bromide, \oplus — \oplus , collagen deaminated and esterified with methyl sulphate, \otimes — \otimes , collagen deaminated and esterified with methyl bromide, \bullet — \bullet , wool esterified with methyl sulphate (from Blackburn *et al* 1941)

Table 3 Soluble nitrogen liberated from methylated collagen after 3 days' exposure to alkaline solutions

Sample	pH of solution	Soluble N (mg/g)	OCH_3 groups removed (g CH_3 /100 g collagen)
Untreated			
C	10.10	2.4	—
C	12.08	3.0	—
C	12.27	3.4	—
C	12.32	3.7	—
C	12.69	4.8	—
C	13.00	6.6	—
A	12.08	3.2	—
After methylation with dimethyl sulphate			
MC_4	7.12	2.6	0.28
MC_4	7.42	4.9	0.29
MC_4	8.60	4.9	0.33
MC_4	8.79	10.4	0.59
MC_4	10.73	15.9	0.98
MC_4	11.80	21.5	1.01
MA_3	12.09	4.9	—
MA_3	12.12	6.3	—
MA_4	12.10	8.3	—
MDA	12.16	5.7	—
After methylation with methyl bromide			
MC_5	8.76	1.1	0.32
MC_5	9.28	1.1	0.27
MC_5	10.20	1.1	0.33
MC_5	11.61	1.6	0.20
MC_5	12.01	1.5	0.34
MC_5	12.24	2.1	0.33

At high pH values loss of methoxyl groups from collagen methylated with methyl sulphate is accompanied by the production of an appreciable amount of nitrogen in the solution (see Table 3)

Collagen methylated with methyl bromide, on the other hand, gave less soluble nitrogen than the untreated collagen. The solutions smelt of methyl amines and gave a positive test for dimethylamine (Dowden, 1938). This smell was also noted by Blackburn *et al* (1941) during the methylation of wool.

It was considered possible that the N in solution was related to the methylation of one of the basic groups of the collagen and specific degradation of this group on treatment in alkaline solution. The arginine content of the collagen as determined by Vickery's method, however, was unchanged by methylation and treatment with alkali, suggesting that the soluble N was not derived from guanidino groups. (There is some evidence, however, that some methylation of guanidino groups does take place. Lindley & Phillips (1947) found no decrease in the arginine content of methylated wool as determined by Vickery's method, but tests on the flavanate precipitate indicated that some change had occurred.) The solution gave only a slight positive reaction for protein, and the results as a whole suggest that the soluble nitrogen is derived from general hydrolysis of the collagen to polypeptides and amino acids.

Titration curves

Deaminated collagen The titration curve of deaminated collagen (DC_2) is given in Fig 2. Deamination has caused a shift in the isoelectric point from 5.5 to 4.5, a decrease in the acid-binding capacity, an increase in the base binding capacity immediately on the alkaline side of the isoelectric point, and a slight increase in the total base bound at pH 12.5. The decrease in acid binding can be attributed mainly to loss of amino groups, but consideration of the free amino N of the original collagen (0.38 mmol/g) shows that at least 0.07 mmol/g of some other basic group has also been affected. The increase in base binding from the isoelectric point to pH 7.0 corresponds to the decrease in acid-binding capacity, and may be attributed to titration of carboxyl groups which have reverted to the uncharged form on the removal of the basic groups. The increase in base binding capacity at pH 12.5 indicates that about 0.18 mmol/g of some group or groups not titrating in the original collagen is now binding base between pH 7 and 12.5.

Methylated collagen The titration curves of collagens MC_2 and MC_4 (methylated with dimethyl sulphate) and MC_5 (methylated with methyl bromide) are shown in Fig 2. In agreement with the hypothesis that methylation involves esterification of ionized carboxyl groups which are the hydrogen ion-fixing groups in the original protein, whilst the basic groups remain charged, the acid-binding capacities of the methylated collagen are decreased by an amount approximately corresponding to their methoxyl contents. The decrease in acid binding capacity, however, is slightly less than the methoxyl content of the collagen, possibly owing to

loss of methyl groups by hydrolysis during contact with the solution. Also, since the isoelectric point of the methylated collagen is about one pH unit higher than that of the original collagen, some of the imidazole groups of histidine which were previously charged, and, therefore, titrated with alkali, will have lost their charge and now contribute to the acid binding capacity.

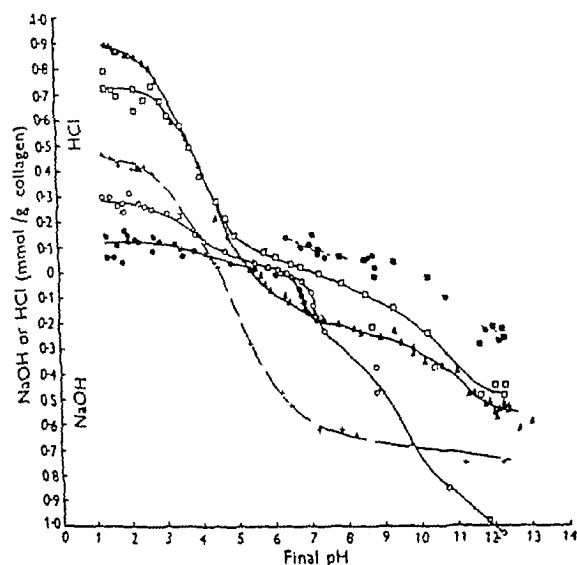


Fig 2 Titration curves of collagen and modified collagens $\Delta-\Delta$, untreated, $+ - +$, deaminated, $\bigcirc-\bigcirc$, esterified with methyl sulphate, CH_3 content 1.04%, $\bullet-\bullet$, esterified with methyl sulphate, CH_3 content 1.04%, \times , corrected for hydrolysis of OCH_3 groups, $\otimes-\otimes$, esterified with methyl sulphate, CH_3 content 1.57%, $\square-\square$, esterified with methyl bromide, CH_3 content 0.35%, $\blacksquare-\blacksquare$, esterified with methyl bromide, corrected for OCH_3 hydrolysis, $\oplus-\oplus$, deaminated and esterified with methyl sulphate, CH_3 content 1.29%

The alkaline section of the curve is more difficult to interpret for, except over a comparatively small pH range, hydrolysis of methoxyl groups takes place with the consumption of alkali. The methoxyl content of the collagens, after contact with the solution, was determined and a correction applied for the alkali consumed, this is only approximate, but the corrected results suggest that appreciably less alkali is bound by the methylated than by the original collagen. In the case of collagen methylated with methyl bromide, the titration curve shows that less alkali is bound even before the correction is applied.

Swelling curves

Deaminated collagen The uptake of water by the deaminated collagen (see Fig 3) differs from that of the original collagen in a manner broadly corresponding to the differences in the titration curves (Fig 2), but at all pH values the water uptake is rather greater than would be expected from consideration of the changes in the reactive groups. This

suggests that deamination has decreased the cohesion of the collagen so allowing it to take up more water.

Methylated collagen In the pH range of stability of the methoxyl groups, i.e. 2–9, the water uptake of methylated collagen is constant, and, when the collagen is fully methylated, is approximately the same as the maximum water uptake of the untreated collagen at pH 2.0 (see Fig 3). This high water uptake over such a wide pH range may be ascribed to the presence of anions in electrovalent association with the basic groups, and consequent setting up of a Donnan membrane equilibrium and a swelling pressure (Donnan, 1911, 1924, Bolam, 1932). In this respect, methylated collagen corresponds to collagen at the point of maximum combination with acid.

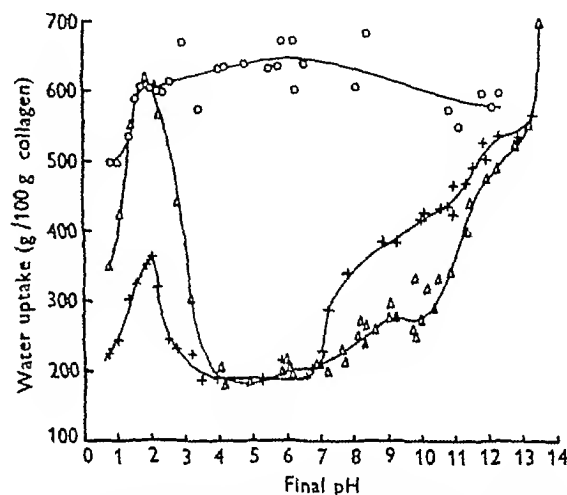


Fig 3 The uptake of water by untreated $\Delta-\Delta$, deaminated $+ - +$, and methylated collagen $\bigcirc-\bigcirc$, at pH values from 0.5 to 13.5

Combination of untreated and modified collagens with tannic acid, mimosa tannins, chromium and formaldehyde

Tannins The conditions of treatment were the same for all the modified collagens, and it is reasonable, therefore, to assume that any differences in the amount of tannin bound are primarily due to modification of the collagen.

Modification of the amino and carboxyl groups of the collagen affects the combination of both tannic acid (hydrolyzable tannin) and mimosa (condensed tannin) with collagen in a similar manner (Figs. 4–6). Deamination decreased the amount of tannin bound at pH values below 4.0, but had little effect at higher pH values, and esterification of the carboxyl groups caused maximum combination of tannin to take place at much higher pH values, and to remain constant at this maximum over a wide pH range, this was especially marked with mimosa tannin (Fig 6).

The addition of 0.5 M-NaCl decreased the amount of tannic acid bound by untreated and deaminated

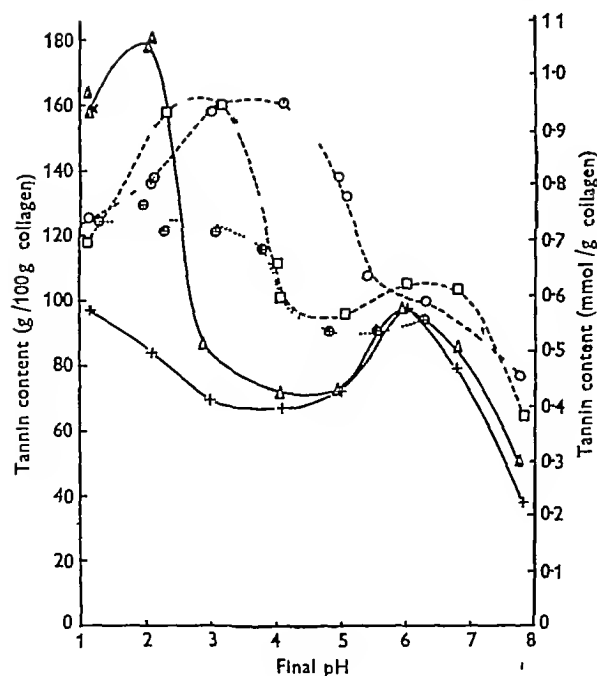


Fig 4 Combination of tannic acid with collagen and modified collagen in the absence of added NaCl $\Delta-\Delta$, untreated, $+ - +$, deaminated, $O-O$, esterified with methyl sulphate, $\square-\square$, esterified with methyl bromide, $\oplus-\oplus$, deaminated and esterified with methyl sulphate

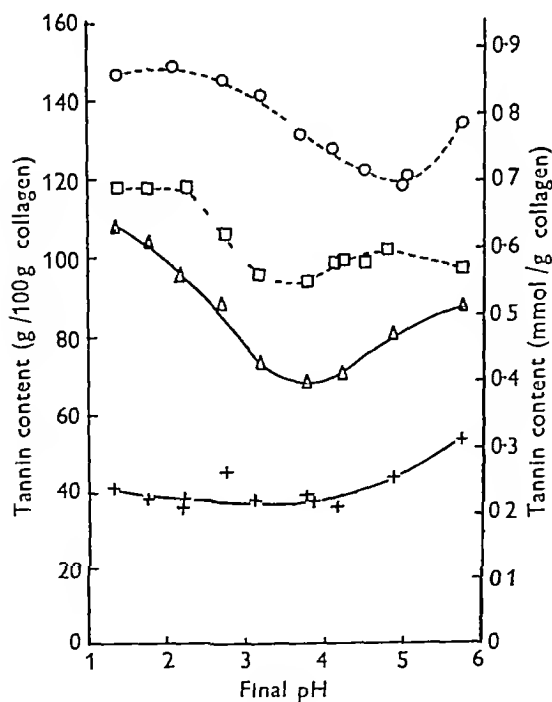


Fig 5 Combination of tannic acid with collagen and modified collagen in the presence of 0.5M-NaCl $\Delta-\Delta$, untreated, $+ - +$, deaminated, $O-O$, esterified with methyl sulphate, $\square-\square$, esterified with methyl bromide

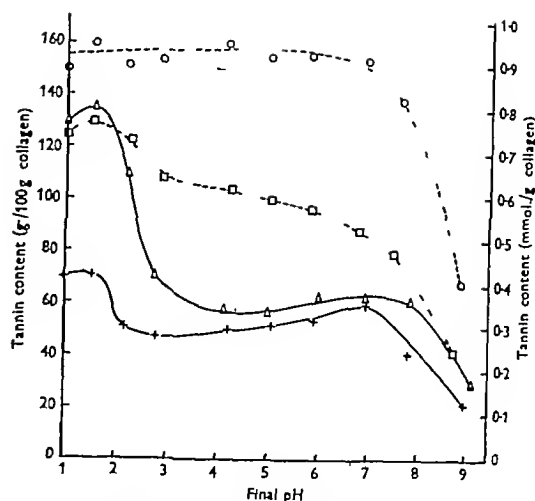


Fig 6 Combination of mimosa tannins with collagen and modified collagen in the absence of added NaCl $\Delta-\Delta$, untreated, $+ - +$, deaminated, $O-O$, esterified with methyl sulphate, $\square-\square$, esterified with methyl bromide

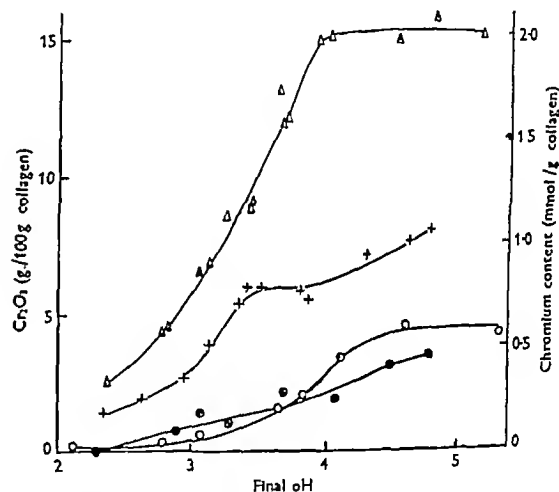


Fig 7 Combination of chromium with collagen and modified collagen $\Delta-\Delta$, untreated, $+ - +$, deaminated, $O-O$, esterified with methyl sulphate, $\oplus-\oplus$, deaminated and esterified with methyl sulphate

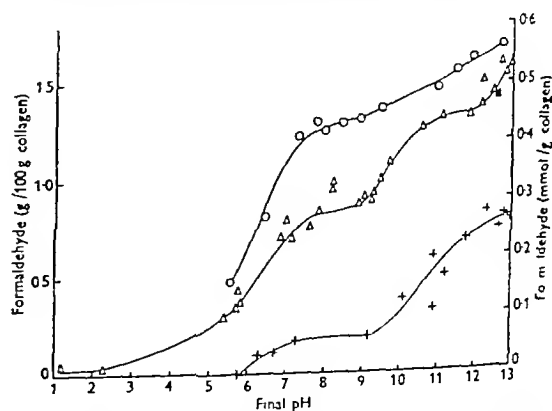


Fig 8 Combination of formaldehyde with collagen and modified collagen $\Delta-\Delta$, untreated, $+ - +$, deaminated, $O-O$, esterified with methyl sulphate

collagen, and by collagen methylated with methyl bromide, especially at low pH values. The tannic acid bound by the collagen methylated with methyl sulphate was only slightly decreased at pH values below 5.0, and at higher pH values was appreciably increased by the presence of NaCl.

The shrinkage temperatures of the collagen and modified collagen were increased from about 60 to between 66 and 84° by combination with tannic acid or mimosa tannin, maximum values in general being obtained when the pH of the tannin solution was between 3.0 and 4.0. The increase in shrinkage temperature was rather greater with mimosa tannin than with tannic acid, possibly because of the lower acidity of mimosa tannin (Hobbs, 1940). The deaminated and methylated collagens tended to have lower shrinkage temperatures than the original collagen treated under the same conditions, and variations of shrinkage temperature with the pH of the tannin solutions were less.

Chromium The chromium bound by the original collagen increased sharply with increase in pH of the chromium sulphate solution up to 4.0, and then remained constant over the pH range studied (Fig. 7). Deamination decreased the combination of chromium over the whole pH range and reduced the maximum amounts combined by approximately half. Methylation decreased combination to a much greater extent. Chromium hydroxide was precipitated on the surface of the methylated collagens at the higher pH values and was difficult to remove, thus, coupled with the possibility that some groups are displaced by chromium during the treatment, makes it not unreasonable to assume that in the absence of carboxyl groups no chromium would be bound by collagen. Deamination of the methylated collagen caused only a further small decrease in the chromium bound. Although the amount of chromium bound by the deaminated collagen was less than that bound by the original collagen, the maximum shrinkage temperature obtained was the same. Treatment with chromium sulphate caused no change in the shrinkage temperature of methylated collagen.

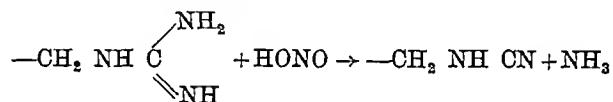
Formaldehyde At pH values below 2.0, negligible amounts of formaldehyde were bound by both the untreated and modified collagens. From pH 2 to 8 the amount bound increased, remained constant between pH 8 and 9, and then increased again at higher pH values (see Fig. 8). Deamination greatly reduced combination between pH 2 and 9. The amount of formaldehyde bound by the untreated collagen up to pH 9.0, and the decrease in combination following deamination, correspond to considerably less than the combination of one formaldehyde molecule by each amino group; similarly, the amount bound from pH 9.0 to 13.0, the upper limit of pH covered, only corresponds to the combination of one

molecule of formaldehyde by each of half the total number of guanidino groups.

With untreated and methylated collagen the combination of as little as 0.4 g formaldehyde/100 g raised the shrinkage temperature to its maximum value (78–80°), whereas the combination of 0.8 g formaldehyde/100 g left the shrinkage temperature of the deaminated collagen unchanged.

DISCUSSION

Deamination Under the experimental conditions employed, treatment with nitrous acid removed almost all the amino groups from collagen and decreased the arginine content by about 20%. The course of the reaction with the amino groups is well known, but little attention appears to have been given to the reaction with the guanidino groups. Bancroft & Belden (1931) and Bancroft & Ridgway (1931) have shown that guanidine reacts with nitrous acid to give cyanamide and ammonia, and it is possible that a similar reaction takes place with the guanidino groups in proteins, namely,



In agreement with this hypothesis, Kanagy & Harris (1935) have shown that ammonia is formed when arginine is treated with nitrous acid, this ammonia will react with nitrous acid to give the excess nitrogen observed in the Van Slyke determination of amino N (Plimmer, 1924; Hunter, 1929; Kanagy & Harris, 1935; Lieben & Loo, 1942; Van Slyke, Hiller & Dillon, 1942). This hypothesis would also explain the higher amide N of the deaminated collagen compared with the original collagen since, under the conditions of the determination, the $-\text{NH}-\text{CN}$ group would probably yield ammonia. This increase in amide N is of the same order as the decrease in the arginine content of the collagen.

The reaction postulated above is also consistent with those changes in the titration curve which cannot be attributed to loss of amino N. Loss of the strongly basic guanidino groups accounts for the decrease in acid-binding capacity; carboxyl groups equivalent to the guanidino groups lost will revert from the zwitterion state ($-\text{COO}^-$) to the uncharged state, and will titrate between the isoelectric point and pH 7.0, and the $-\text{NH}-\text{CN}$ groups, being feebly acidic, will titrate between pH 7 and 12, thus accounting for the increased base-binding capacities in these ranges. Since guanidino groups do not titrate with base up to pH 12.5 (Bowes & Kenten, 1948a), the total base-binding capacity should be increased by an amount equal to twice the $-\text{NH}-\text{CN}$ groups formed, i.e. base bound by the additional

un-ionized carboxyl groups plus that bound by $-\text{NH CN}$ groups. On this basis, and assuming that 0.05 mmol/g amino groups remain (see Table 1), the curve indicates that about 0.10 mmol/g guanidino groups are converted to cyanide groups (Decrease in acid binding in excess of that due to amino groups, 0.12 mmol/g, base bound between 7 and 12, 0.10 mmol/g, and total increase in base-binding capacity, 0.20 mmol/g). This figure is in good agreement with decrease in arginine content found by analysis, 0.09 mmol/g.

Esterification Like the carboxyl groups of amino-acids, those of wool, silk, gelatin, and collagen can be esterified with methyl sulphate and methyl bromide (Blackburn *et al* 1941, Blackburn & Phillips, 1944).

The evidence obtained in the present investigation confirms this view with respect to collagen. The reaction would appear to necessitate the presence of basic groups equivalent to carboxyl groups, it was found, however, that deamination did not decrease the extent of methylation. Blackburn & Phillips (1944) considered that the lysine content of wool is so low that its removal does not materially affect the number of basic groups. This explanation is not applicable to collagen since amino groups form about one third of the total basic groups, and a more likely explanation is that as ionized carboxyl groups are esterified, further carboxyl groups ionize under the influence of the guanidino groups, so that eventually all carboxyl groups are esterified.

Consideration of the values for the free carboxyl groups in the untreated collagens, as indicated by analysis and by their titration curves (Bowes & Kenten, 1948b), shows that all the methoxyl groups introduced can be accounted for on the basis of esterification of carboxyl groups. In three experiments, the number of methoxyl groups was slightly in excess of the number of free carboxyl groups but this excess was within the experimental error. Blackburn *et al* (1941) and Blackburn & Phillips (1944) found that the methoxyl groups introduced by methyl sulphate exceeded the number of free carboxyl groups believed to be present in wool, silk, gelatin, and collagen, and suggested that methylation also occurred at certain 'activated' peptide links. It is now known (Bowes & Kenten, 1948a) that the values taken for the number of carboxyl groups in gelatin and collagen were low, and the present work shows that with collagen there is no necessity to postulate any form of *O*-methylation other than that of carboxyl groups. It is possible that future determinations of the dicarboxylic acids in wool and silk will obviate the necessity of assuming that these proteins undergo peptide methylation.

The low amino nitrogen values of the methylated collagens suggest that some *N*-methylation has occurred, especially with methyl bromide. The lower

base-binding capacity of the methylated collagens as compared with that of untreated collagen may also be due to *N*-methylation, for although the introduction of a methyl group will not eliminate the basic characteristics of the amino group, it will, by analogy with the methylamines, increase the *pK* of these groups and hence cause a shift in the titration curve to higher pH values. It is possible that the titration curves of the methylated collagen would show the same maximum base binding capacity as the original collagen if carried to higher pH values. It is also possible that the reduction in base binding capacity of the collagen methylated with methyl sulphate is related to the presence of nitrogen in the solutions after contact with the collagen, if this nitrogen were present as a base (for instance, as methylamine) it would titrate and so reduce the apparent fixation of base by the collagen.

Combination of collagen and modified collagens with tannins, chromium and formaldehyde Although tannic acid, in contrast to the tannins of mimosa, contains acidic groups (Sunthakar & Jatker, 1938, Abichandani & Jatker, 1938, Cheshire, Brown & Holmes, 1941) which might be expected to form salts with the basic groups of the collagen, the mode of combination with collagen appears to be the same with both materials. The results as a whole are consistent with the supposition that combination of tannin is related to the positive charge carried by the protein. With the untreated collagen, combination is greatest between pH 1.5 and 2.0 when the collagen carries its maximum net positive charge, decreases as the pH increases and the net positive charge decreases, and eventually is reduced to negligible proportions at pH 8.0–9.0 when the basic groups begin to lose their positive charge and the protein carries a net negative charge. Deamination which decreases the positive charge on the collagen, decreases combination of tannin, and esterification, which causes the collagen to carry its maximum positive charge over the whole pH stability range of the methoxyl groups, causes combination of tannin also to be at a maximum over this range. Experiments indicate that the molecular weight of tannic acid and mimosa tannin is 1700 (Brintzinger & Brintzinger, 1931, Humphreys & Douglas, 1937), and the equivalent weight of tannic acid is of the same order (Cheshire *et al* 1941). Using these figures, an interesting comparison can be made between the amounts of tannin bound and the acid binding capacity of the collagens (see Table 4). The molecular amounts of tannin combined with the untreated and deaminated collagens between pH 1.5 and 2.0 are of the same order as their acid-binding capacities, deamination halves the acid-binding capacity and also halves the amount of tannic acid and mimosa tannin bound. This suggests that one molecule of tannin is associated with each basic

group Although the amounts of tannin bound are dependent on the concentration of tannin in the solution, the time of treatment and method of washing, it would seem reasonable to assume that there is some significance in these stoicheiometric relationships

Table 4 *Acid and tannin bound by collagen and deaminated collagen*

(Results in mmol /g)

Collagen	Acid binding capacity	Tannin bound between pH 1.5 and 2.0	
		Tannic acid	Mimosa tannin
Untreated collagen A	0.88	1.0	0.80
Deaminated collagen DA ₁	0.44	0.50-0.55	0.41

The present results emphasize the essential part played by the carboxyl groups in the binding of chromium, and strengthen the current view that the fixation of metals involves complex formation with the carboxyl groups of the protein in a manner analogous to the complex formation which occurs with the carboxyl groups of organic acids (for review of literature see Bowes, 1948). The amino groups also appear to be involved since deamination decreased the amounts bound by the untreated collagen, though not by the methylated collagen. These findings are consistent with the hypothesis that combination of chromium involves co-ordination of both carboxyl and amino (or other basic groups) with the same chromium complex. A similar hypothesis has been put forward to account for the fixation of calcium by proteins (Greenberg, 1944), and for the high thermal stability of chrome-tanned leather (Kuntzel & Riess, 1936).

Although the validity of stoicheiometric relationships between the amounts of chromium bound and the reactive groups of the collagen may, in view of the complexity of the system, be open to question, it is of interest to note that the amount of chromium bound between pH 4 and 5 approximately corresponds to the fixation of two chromium atoms by each carboxyl group, and the decrease in combination caused by treatment with nitrous acid corresponds approximately to two chromium atoms for each basic group lost. It is probable that the chromium aggregates in the tanning solutions used contained, on the average, two chromium atoms (Bowes, 1948).

The evidence suggests that, in collagen, the amino and guanidino groups are the main centres involved in the binding of formaldehyde, and there is no evidence that the amide groups are concerned under

the conditions employed. The importance of the amino groups raising the shrinkage temperature (Gustavson, 1943) is confirmed. Free amino groups also play an essential part in the hardening of casein with formaldehyde (Nitschmann & Hadorn, 1944, Nitschmann & Lauener, 1946), and it is probable that a similar mechanism is involved, namely, the formation of cross links between adjacent polypeptide chains. Nitschmann & Hadorn (1944) and Nitschmann & Lauener (1946) discuss the various ways in which such cross links may be formed and consider that a cross link between ϵ -NH₂ groups and —NH— groups of the peptide link is the most probable. (For further discussion on this point see Fraenkel-Conrat, Cooper & Olcott, 1945, French & Edsall, 1945, Bowes, 1948).

SUMMARY

1 Collagen has been deaminated with nitrous acid and esterified with methyl sulphate and methyl bromide, and the effect of these treatments on the reactivity of collagen towards acids, bases, tannins, chromium and formaldehyde has been determined. Modification of the guanidino groups by treatment with hypochlorite (Sakaguchi (1925) reaction) was only partially successful, only 40-50 % of the arginine was destroyed and there was extensive general breakdown of the collagen.

2 Treatment with nitrous acid removed almost all the amino groups from collagen and decreased the arginine content by approximately 20 %. Evidence deduced from analysis and titration curves suggests that the guanidino groups are converted into cyanamide groups.

3 All the O-methyl groups introduced into collagen by methyl sulphate and methyl bromide can be accounted for on the basis of esterification of carboxyl groups. There is an indication that some N methylation occurs, especially with methyl bromide.

4 From a study of the combination of tannins, chromium and formaldehyde with untreated, deaminated and methylated collagen, it is suggested that combination of tannins is related to the positive charge carried by the collagen, combination of chromium involves co-ordination of both amino and carboxyl groups of the collagen with the same chromium complex, and combination with formaldehyde occurs mainly with the amino and guanidino groups. Increase in thermal stability results only from combination of formaldehyde with amino groups.

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The Behaviour of *Lactobacillus arabinosus* towards Nicotinic Acid and its Derivatives

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These investigations originated in studying the basis of a method for microbiological assay of nicotinic acid (McIlwain & Stanley, 1948). The method was that in which acid produced from glucose by *Lactobacillus arabinosus* 17-5 is titrated with alkali after a period of incubation of about 3 days. The assay depends on the empirical observation that the quantity of titratable acid produced during incubation is dependent on the quantity of added nicotinic acid. It was found (McIlwain, 1948) that this relationship existed because of changes which took place in nicotinic acid during the assay, the compound was first assimilated to, and later lost from, the cells. Its loss was found to be conditioned by its functioning as a catalyst, and was such that nicotinic acid exhibited a defined catalytic capacity, measured by the ratio titratable acid formed/nicotinic acid lost ($\mu\text{mol/mol}$). Values of the catalytic capacity (about 5×10^5) were relatively independent of the intensity with which nicotinic acid derivatives were functioning as catalysts, which was expressed as a catalytic activity ($\mu\text{mol titratable acid/hr/mol nicotinic acid}$, values ranged from 5 to $15 \times 10^3 \text{ hr}^{-1}$).

Such observations suggested the need for more detailed investigations of the form in which nicotinic acid derivatives existed in the bacterial cells. We have now examined the nicotinic acid of cells of *Lb arabinosus*, and found it to be largely in the form of cozymase. *Lb arabinosus*, in marked distinction from a number of other organisms, reacted only slowly with added cozymase, this, and our interest in the practical problems presented by microbiological assay, directed our attention to metabolic changes occurring in the cozymase of the bacterial cells, and these are described below.

EXPERIMENTAL

The assays for nicotinic acid, the preparation of growth media and of materials for assay, and the handling of non-proliferating suspensions of *Lb arabinosus* were carried out as described by McIlwain (1948). Determination of V-factor and of cozymase, and the extraction of these substances from bacteria were made by the methods used by McIlwain & Hughes (1948).

RESULTS

Assimilation of nicotinic acid by Lactobacillus arabinosus

1 *Cell content* Cells of *Lb arabinosus* from cultures whose growth has not been limited by nicotinic acid may, nevertheless, contain varying quantities of this substance. This is shown in Table 1. Nicotinic acid becomes a limiting factor in growth of the organism at concentrations of about $2 \times 10^{-7} \text{ M}$ and the range used in assay is below $1.5 \times 10^{-7} \text{ M}$. The values of Table 1 illustrate also the previous findings (McIlwain, 1948) that the nicotinic acid content of the cells of a given culture falls with time. To approach saturation of the cells with the acid at 26 hr in the relatively early part of a culture's growth, a concentration of c. $5 \times 10^{-7} \text{ M}$ nicotinic acid is seen to be required. The cells will thus be unsaturated in this sense during most of their period of incubation in all the concentrations used in assay.

Under the conditions most favourable for assimilation, about 80% of the nicotinic acid added to a culture has been recovered from the cells after 26 hr growth. As the cells inactivate nicotinic acid during this period, this result gives a minimum estimate only of the completeness with which they can assimilate the acid. The actual concentration of nicotinic acid derivatives in the cells after assimilation can be calculated from the data of Table 1 to be about 2 to $10 \times 10^{-4} \text{ M}$. In making this calculation the wet weight of the cells was taken as five times their dry weight, this has been found to be a typical ratio. The values recorded in Table 1 for the yield of bacterial cells from unit volume of culture show that the assay medium, though carefully chosen for maximum response to limited nicotinic acid, does not give high yields of bacteria.

2 *Nature of the assimilated nicotinic acid* Most of the nicotinic acid of the cells existed as derivatives, especially in cells containing limiting quantities of the substance. Table 2 A shows that some 90% of the acid, determined in cells grown from a concentration of the acid within assay range, exists as

Table 1 *Nicotinic acid content of cells of Lactobacillus arabinosus after growth of varying types*

Nicotinic acid of growth medium ($\mu\text{M} \times 10^{-7}$)	Period of incubation (hr)	Yield of cells (mg dry wt / 100 ml culture)	Nicotinic acid content of cells		
			(μmol /cells of 100 ml medium)	(μmol /mg dry wt)	In fresh cells* ($\text{M} \times 10^{-4}$)
1.2	30	7.0	6.2	0.88	1.8
2.4	26	10.4	13.9	1.33	2.7
4.9	26	9.5	37	4.04	8.1
4.9	42	10.7	33	3.06	7.1
4.9	48	12.0	28	2.34	4.7
12.2	26	9.8	53	5.4	10.8

* Calculated, assuming a water content of 80 %

Organisms were grown in quantities of 200–500 ml of the assay medium (cf McIlwain & Stanley, 1948) with nicotinic acid added in the quantities indicated

Table 2 *Nicotinic acid derivatives in cells of Lactobacillus arabinosus, and in material liberated from cells to solution*

Nicotinic acid in growth medium (μmol /100 ml)	Period of incubation in growth (hr)	Yield of cells (mg dry wt / 100 ml culture)	Later treatment	Content (μmol /cells of 100 ml culture)		
				Nicotinic acid	V factor	Cozymase
A Material in cells						
12.2	30	13.0	Washed	6.9	—	6.5
12.2	30	13.0	Washed, and in saline mixture* at 37°, 6 days	2.7	—	2.4
4.5	28	10.9	Washed	19	14	—
12.2	48	12.8	Washed	43	27	—
B Material liberated on exposure of cells in salt mixture†						
4.5	28	10.9	Cells washed and	6.1	4.2	—
11.2	48	12.8	exposed†	16.4	8.4	—

* The inorganic salts of the medium of Barton Wright (1946) at pH 6.8 and in the concentrations of the final assay medium

† In the inorganic salts (organisms from 100 ml of culture in 50 ml salt solution) for 72 hr at 37°, aerobically

cozymase. The amount determinable as V-factor (probably equivalent to nicotinamide ribosides for discussion see McIlwain, 1947) was also high.

3 *Relative stability of cozymase added to Lb arabinosus*. McIlwain & Hughes (1948) found that many bacterial species rapidly inactivate added cozymase, even at low concentrations of cozymase ($c 10^{-5}$ M) loss may occur at the rate of 10 μmol /mg dry wt /hr, and with higher concentrations may rise to over 500 μmol /mg /hr. Such a coefficient means that the organism is inactivating its own weight of cozymase in about 3 hr. *Lb arabinosus* was, however, relatively inert towards cozymase. Under the conditions of its use in assay, i.e. with cells of between 1 and 3 days growth, and at pH values between 5 and 7, any reaction with 10^{-5} or 10^{-4} M-cozymase was of $< 1 \mu\text{mol}$ /mg dry wt of cells/hr. In attempts to observe a more rapid reaction, experiments were also carried out at pH 4.5 and 7.0, in the presence and absence of phosphates and of glucose, and with some suspensions initially rich and others initially poor in nicotinic acid derivatives. Again, any reaction was of $< 1 \mu\text{mol}$ /mg /hr. It was evident that *Lb arabinosus* did not inactivate cozymase at the rapid rate observed in several other bacteria. Neverthe-

less, slow inactivation has been observed (Table 1), and found to be related to the assay (McIlwain, 1948); this process has, therefore, been studied further.

Change in nicotinic acid in suspensions of Lactobacillus arabinosus

An attempt was made to reproduce the changes of Table 1 under conditions better defined than those of a growing culture. Table 3 shows that the cellular nicotinic acid of *Lb arabinosus* can also be very stable. Cells were examined which had been grown in media relatively rich and relatively poor in nicotinic acid, the latter conditions (12 μmol nicotinic acid/100 ml) are within the range used in assay. In all cases the cells could be incubated at 37° in salt mixtures at pH 6.5–7 for 6 days without any loss in the total nicotinic acid of the suspension. A small increase, of some 10% of the initial value, was frequently found.

These experiments showed that the loss in nicotinic acid observed during the assay was not brought about by the bacteria when they were incubated in the presence of the majority of the constituents of the assay medium. The components omitted from the suspending fluids during the

experiments quoted were glucose, the casein hydrolysate providing amino-acids to the medium, and in one case sodium acetate. Other results in Table 3 showed sodium acetate to have only a small effect in the change in nicotinic acid, but addition of glucose caused a major change. In each case examined, addition of glucose led to a decrease in nicotinic acid content.

The course of changes in nicotinic acid derivatives in suspensions of Lactobacillus arabinosus

1 *In absence of glucose* Although the total nicotinic acid of cell suspensions changed little under these conditions, marked changes occurred in its distribution. Fig 1 shows this in the case of a suspension of cells which were initially rich in the

Table 3 *Changes in the nicotinic acid derivatives of suspensions of cells of Lactobacillus arabinosus*

Batch of organisms, conditions of growth	Initial content of nicotinic acid ($\mu\text{mol}/\text{mg}$ dry wt)	Conditions of reaction	Change in nicotinic acid during 6 days* ($\mu\text{mol}/\text{mg}$ dry wt)
1 26 hr, 122 μmol nicotinic acid/100 ml	5.4	Salts of medium, † pH 6.8	+0.70
		As above, with glucose (0.1 M)	-1.80
2 30 hr, 12 μmol nicotinic acid/100 ml	0.88	Salts of medium, pH 6.5	+0.10
		As above, with glucose (0.1 M)	-0.25
3 26 hr, 24 μmol nicotinic acid/100 ml	0.72	Inorganic salts of medium, ‡ pH 7	+0.08
		As above, with glucose (0.1 M)	-0.14

* The change quoted is in the whole reaction mixture, the kinetics of the change and the partition of nicotinic acid between cells and solution are discussed later.

† NaCl, Na acetate, and inorganic salts A and B of Barton Wright (1946), at the concentrations used in assay.

‡ As in footnote †, but lacking Na acetate.

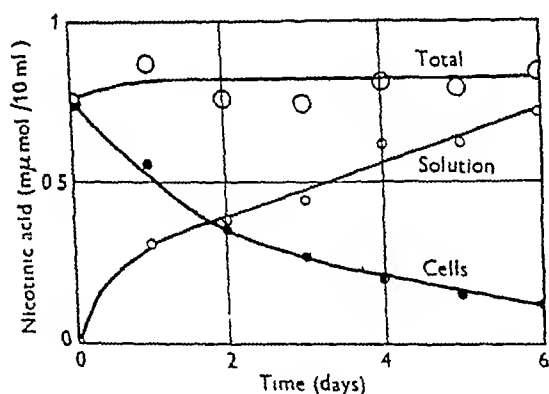


Fig 1

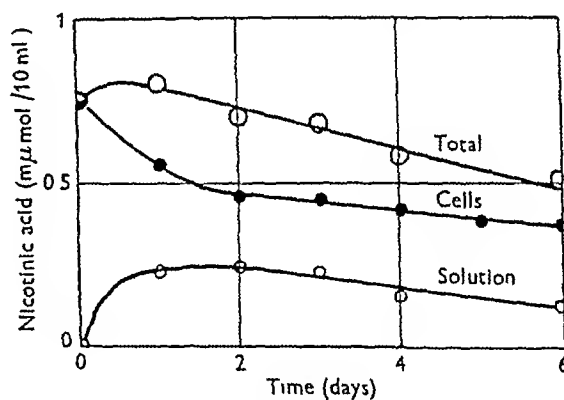


Fig 2

Figs 1 and 2 Change in distribution of nicotinic acid in suspensions of cells of *Lb arabinosus*, grown with 122 μmol of the acid/100 ml. Cells were harvested after 26 hr growth, and washed with, and suspended in, the inorganic salts of the growth medium without glucose (Fig 1) and with 0.1 M glucose (Fig 2).

Although the effect of glucose was marked, the absolute magnitude of the change which it caused was small. It led to loss of about 30% of the cells' nicotinic acid in 6 days, but this involved an average rate of loss over that period of only 2-7 $\mu\text{mol}/\text{mg}$ dry wt of cells/hr. The change in a batch of cells grown from 12 μmol nicotinic acid/100 ml, and thus within the assay range, was about -2.5 $\mu\text{mol}/\text{mg}$ /hr, and thus much slower than that estimated as occurring during assay. This led us to investigate the course of the change, the distribution of nicotinic acid between cells and solution, and the effect of additional substances on the changes.

The quantity in the cells fell throughout the experiment, but at a decreasing rate, and the acid which was lost from the cells appeared in the solution. Results similar to those of Fig 1 have been obtained also with cells which were initially poorer in nicotinic acid (containing 30 or 15 $\mu\text{mol}/100$ ml, the latter value being the upper limit of the assay range), but in such cases the proportion of nicotinic acid leaving the cells was smaller than with those initially rich in the acid.

The nicotinic acid-containing material liberated from the cells consisted at least in part of derivatives of the acid. The solutions yielded were too dilute to give significant values for cozymase in the apozym-

mase system, but their V-factor activity could be determined. The values obtained (Table 2B) showed two thirds or one half of the liberated material to possess such activity and presumably to be at least of the complexity of a riboside of nicotinamide.

2 *In presence of glucose* Glucose led not only to the decrease in total nicotinic acid noted in the previous section, but in many cases it also caused

(1.22 $\mu\text{mol}/10\text{ ml}$) within the assay range and were reaped at 30 hr when their rate of acid formation was high. Their initial rate in the salts of the assay medium, with glucose, was $> 10\ \mu\text{mol}/10\text{ ml/hr}$, and this fell to about $1\ \mu\text{mol}/10\text{ ml/hr}$ on the sixth day. The rate fell rapidly during the first 2 or 3 days and then more slowly. Changes in the rate have, therefore, been examined in relation to changes in cellular nicotinic acid.

Table 4 *Effect of glucose on distribution of nicotinic acid in suspensions of Lactobacillus arabinosus*

Conditions of growth and reaction	Nicotinic acid ($\mu\text{mol}/10\text{ ml}$ of suspension)			Percentage of nicotinic acid present in cells after 6 days
	Initially	After 6 days		
		Cells	Solution	
As 1, Table 3, no glucose	4.9	0.81	4.8	14
As 1, Table 3, with glucose	4.9	2.44	0.9	73
As 2, Table 3, no glucose	3.05	1.06	2.26	32
As 2, Table 3, with glucose	3.05	1.38	1.0	58
As 3, Table 3, no glucose	0.88	0.49	0.45	52
As 3, Table 3, with glucose	0.88	0.29	0.26	53

a marked change in the distribution of nicotinic acid derivatives between cell and solution. Fig. 2 shows that, with organisms rich in nicotinic acid, glucose caused nicotinic acid which otherwise would have passed into solution to be retained in the cells. The quantity in solution increased only transiently, and then fell in a fashion roughly parallel to that of the cells. This behaviour was less marked in organisms which initially contained less nicotinic acid, and with cells grown from 12 μmol of the acid/100 ml no considerable change in distribution of the acid was brought about by glucose (Table 4), but the fall in total quantity remained marked.

Acid formation from glucose by suspensions of Lactobacillus arabinosus

1 *Fall with time* The changes in cellular nicotinic acid are relevant to the assay because this depends on acid formation by the cells, and this again depends on the presence of nicotinic acid derivatives. It has already been shown (McIlwain, 1948) that freshly harvested cells of *Lb. arabinosus* are capable of fermenting glucose at rates almost equal to the most rapid reached during growth in nutritionally rich media. Organisms from nicotinic acid-deficient cultures were found to produce acid at a lower rate than those with excess of the acid, and the deficiency could be made good by addition of nicotinic acid or cozymase. In these experiments acid formation was followed for only an hour or two after harvesting.

The course of acid formation from suspensions of *Lb. arabinosus* was now followed over periods comparable to those of the assay. Fig. 3 shows that observed in an experiment lasting 6 days. Organisms had been grown from a quantity of nicotinic acid

2 *Catalytic activity of nicotinic acid in cell suspensions* Relevant results are presented in Table 5. Here the rate of acid formation in the suspensions is expressed as a multiple of the quantity of nicotinic acid in the cells. These values (catalytic activities in $\text{mol titratable acid/mol nicotinic acid/hr}$) are seen to be relatively constant but to fall during the course of exposure of the cells. The cells are thus changing

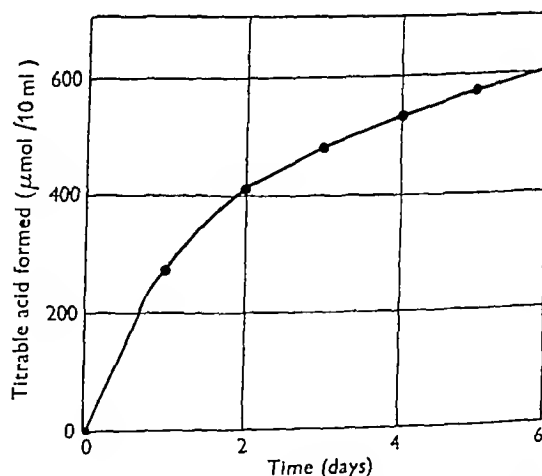


Fig. 3 Course of formation of titratable acid in suspensions of *Lb. arabinosus*, grown in the same way as the organisms of Figs 1 and 2 but for 30 hr, and suspended in the medium described for Fig. 2.

during exposure so that nicotinic acid becomes progressively less effective as a catalyst. It is also initially much less effective as a catalyst during the present experiments than in assay; the catalytic activities of Table 5 are only about one tenth of those found (McIlwain, 1948) at comparable pH values during assay.

The higher catalytic activity during assay did not appear to be due to cell proliferation, as the earlier experiments included ones of short duration with harvested and resuspended cells. The difference was found to be due to the organic constituents of the suspending fluid. Reconstituting the assay medium by adding to the suspending fluid of Table 5 casein hydrolysate, ammonium salts, and a mixture of vitamin-like substances restored the catalytic activity to 14,000 mol/mol/hr at pH 6, or 8000 at pH 5.2. In this effect the vitamin-like substances played little part (Table 5).

their cells. The assimilated material has been found to be present largely as cozymase, which is much more stable when added to suspensions of *Lb arabinosus* than it is in suspensions of many other organisms. Loss of cozymase from buffered suspensions of *Lb arabinosus* was extremely slow, both with respect to cozymase which was added as such, and also to that which was synthesized by the cells themselves from nicotinic acid. Cellular cozymase became markedly less stable in glycolyzing organisms although here also the rate of its loss was by most standards very low, being about 10 $\mu\text{mol}/\text{mg}$ dry

Table 5 *Catalytic activity and catalytic capacity of nicotinic acid in acid formation by suspensions of Lactobacillus arabinosus*

(Cells were grown with 1.2 μmol nicotinic acid/10 ml, reaped after 30 hr incubation, washed twice aseptically with, and suspended in, the inorganic salts and buffers of the assay medium together with glucose but excluding the ammonium salts. Initial pH of medium 6.5, concentration of cells 2.95 mg/10 ml.)

Age of suspension (days)	pH	Nicotinic acid content of cells ($\mu\text{mol}/10\text{ ml}$ suspension)	Rate of formation of titratable acid ($\mu\text{mol}/10\text{ ml}$ suspension/hr)	Catalytic activity (mol titratable acid/mol nicotinic acid/hr)	Catalytic capacity (mol titratable acid formed/mol nicotinic acid lost) ($\times 10^5$)
1	5.7	2.44	9.1	3700	3.5
2	5.4	2.04	4.6	2300	2.1
3	5.3	1.70	2.62	1500	2.3
4	5.25	1.48	2.04	1400	2.0
5	5.2	1.28	1.52	1200	2.1
6	5.2	1.14	1.08	1000	

A parallel experiment was carried out with similarly grown organisms in a suspending fluid of the same initial pH but with in addition the aneurin, riboflavin, pyridoxin, pantothenate, p-aminobenzoic acid and biotin of the assay medium. Catalytic activities of 2000–1200 were observed, and catalytic capacities of $2.5\text{--}3.5 \times 10^5$ (mean, 2.6×10^5).

3 *Catalytic capacity of nicotinic acid in cell suspensions*. In Table 5 are given also the ratios between the mol titratable acid formed and the mol nicotinic acid lost. These values (the catalytic capacities of nicotinic acid) were relatively steady during the experiments and were not far removed from the values observed in assay. Thus, different suspensions gave $2.5\text{--}3.5$ and $2.0\text{--}3.5 \times 10^5$, and in assay the capacity was about 5×10^5 . It has been observed above that in the present experiments, as in assay, loss of nicotinic acid was associated with the presence of glucose. The values now quoted show that the quantitative relations between the metabolism of glucose and of nicotinic acid are also similar during the assay in non-proliferating suspensions, although the absolute rates of metabolism of each fell in the suspensions to only a fraction of their value in assay.

DISCUSSION

The present studies have given further information on the behaviour of nicotinic acid in *Lactobacillus arabinosus*, and this behaviour may now be assessed in relation to the use of the organism in assay. The organisms, in growth, assimilated nicotinic acid from very dilute solutions and concentrated it in

wt/hr. It was, therefore, very interesting to observe that this rate bore a relation to the rate of glycolysis similar to that previously observed in more active cells during assay. In both cases the catalytic capacity of nicotinic acid, or the ratio between the rates of change in titratable acid in nicotinic acid, was between 2 and 6×10^5 . The possible significance of this has been discussed previously (McIlwain, 1948).

These values give another instance of relative stability in the catalytic capacity of nicotinic acid during marked changes in its catalytic activity (i.e. the rate of reaction which unit quantity of it catalyzes, in this case glycolysis, expressed in $\mu\text{mol}/\text{hr}/(\mu\text{mol}$ nicotinic acid). The catalytic activity was markedly affected by the suspending fluid. This is understandable, as glycolysis involves many reactions, and the slowing of any one of these during the 6 days of an experiment could be reflected in the overall rate of reaction. The relative constancy of the catalytic capacity implies that, when glycolysis has fallen in rate, loss of nicotinic acid has also been correspondingly slower.

Data already available from various assay methods for nicotinic acid give further indications of the relative constancy of the catalytic capacity of the acid. As emphasized previously (McIlwain, 1948), the full catalytic capacity of nicotinic acid is

not used during an ordinary assay. In the instance investigated, normal assay practice, which allows reaction to continue until acid formation becomes slow, employs about half the full catalytic capacity of the added nicotinic acid. The standard dosage/response curve of assay, when expressed in mol titratable acid/mol nicotinic acid, thus gives a partial catalytic capacity of nicotinic acid. Values of this type have been collected in Table 6. This shows partial catalytic capacities from our own observations with *Lb arabinosus* to range from 2.6 to 4.8×10^5 , those of other observers with this organism, from 1.7 to 2.6, and with *Leuconostoc mesenteroides*, from 4.9 to 6.8×10^5 . Fundamental importance is not

0.7–5 $\mu\text{mol}/\text{mg}$ dry wt of cells, according to conditions of growth.

2 The assimilated substance existed in the cells largely as cozymase. Cozymase in *Lb arabinosus*, or added to suspensions of this organism, was much more stable than in the presence of many other bacteria.

3 A slow reaction (of 2–20 $\mu\text{mol}/\text{mg}/\text{hr}$), leading to inactivation of cozymase and of its nicotinic acid moiety did, however, occur in *Lb arabinosus* during glycolysis. Glycolysis altered also the distribution of nicotinic acid between saline solutions and the cells of the organism, leading to greater retention of the acid in the cells.

Table 6 Partial catalytic capacities of nicotinic acid during assays

Source of data	Conditions of assay		Partial catalytic capacity $\times 10^5$ *
	Organism	Temp. of incubation	
12 assays in conjunction with present paper, and that of McIlwain (1948)	<i>Lactobacillus arabinosus</i>	37	2.6–4.8, mean, 3.4, standard deviation, 0.65
Barton Wright (1946)	" "	30 or 37	2.6
Snell & Wright (1941)	" "	30	2.2
Roberts & Snell (1946)	" "	37	1.7
Krehl, Strong & Elvehjem (1943)	<i>Leuconostoc mesenteroides</i>	30	4.9
Johnson (1945)	" "	30	6.75

* Molar ratio: titratable acid formed/nicotinic acid added, during the approximately linear portion of the dosage/response curve. Different media of growth were employed by the different investigators, titratable acid was in all cases determined after 3 days' growth.

attributed to the partial catalytic capacities which are expressed immediately by these values, but the latter are considered to indicate that the catalytic capacities themselves are also likely to be of the same order of magnitude under the various conditions chosen by the different observers whose results have been given in Table 6. This increases the significance of the catalytic capacity.

SUMMARY

1 *Lactobacillus arabinosus* 17–5, which is used in a microbiological assay of nicotinic acid, assimilated this acid from 10^{-8} or 10^{-7} M solutions during growth. Its cell content of the substance was then

4 Rates of glycolysis and of loss in nicotinic acid varied in parallel in suspensions of *Lb arabinosus*, as they had previously been found to do in assay. The ratio between the two rates (the catalytic capacity of nicotinic acid) had approximately the same value, of $3\text{--}6 \times 10^5$, in the two cases.

5 On the other hand, the activity of nicotinic acid as a catalyst was much lower in non-proliferating suspensions in simple media, than in assay. Catalytic activity and partial catalytic capacity have been used as measures of the behaviour of the organism during assays of the present type.

We are greatly indebted to Miss E. Ellis for assistance during these investigations.

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Distribution of Glutamine and Glutamic Acid in Animal Tissues

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It is generally accepted that glutamine and glutamic acid, apart from serving as structural units in proteins and peptides, play a special role in the metabolism of animals, plants and micro organisms. A few specific functions have already come to light (see the reviews of Archibald, 1945, 1947), but it is probably correct to say that the chief functions are still unknown. It was thought that a survey of the occurrence of glutamine and glutamic acid in biological material might assist in elucidating the part played by the two substances in metabolism, and the two substances were, therefore, determined in a number of animal tissues. Surveys of the distribution of glutamine have been made by previous workers (Ferdman, Frenkel & Silakova, 1942, Hamilton, 1945), but data on the glutamic acid content of tissues are scanty because, until recently, no specific and convenient methods applicable to small quantities of material were available.

EXPERIMENTAL

The procedure described recently (Krebs, 1948) was used. All tissues except blood were frozen in liquid air as soon as possible, usually within 2 or 3 min after death. A delay of about 5 min was unavoidable in the case of sheep brain and of about 20–40 min in the case of foetal material. A quantity of about 5 g, in some cases less, was weighed out in the frozen state, and crushed in a mortar with 2 vol of 0.5N HCl and washed sand. Blood was collected from fasting hospital patients, mixed with heparin and immediately centrifuged. The plasma (10 ml) was shaken *in vacuo* after addition of 0.25 ml N HCl to remove bicarbonate and CO₂. Two Warburg flasks were used for one analysis, both containing 4 ml of plasma and 0.3 ml of 3M acetate buffer pH 4.9 in the main compartment and one containing 0.5 ml of washed bacterial cells in the side arm.

RESULTS

Glutamine and glutamic acid in animal tissues

Results obtained on various tissues, excluding blood and foetal tissues, are shown in Table 1. All tissues examined contained considerable quantities of glutamic acid and in most glutamine was also present. The sum of glutamic acid and glutamine in different samples of the same tissue varied less from animal to animal than the concentrations of the two components. In most tissues the concen-

tration of glutamic acid + glutamine was much greater (c. 10 times) than the concentrations in the blood plasma, exceptions being adipose tissue, the crystalline lens and vitreous humour, all tissues whose metabolic activities are in general low. The highest concentrations were found in brain, mammalian heart and spleen (average concentrations between 10 and 15×10^{-6} mol/g or 146–220 mg/100 g). Relatively low values (average below 5×10^{-6} mol/g or 73 mg/100 g) were found in ovary, thyroid, lung and the tissues already given as having concentrations of the same order as blood plasma. Intermediate values (average between 5 and 10×10^{-6} mol/g or 73–146 mg/100 g) were found in the other tissues tested.

The proportion of glutamic acid to glutamine showed consistent differences from tissue to tissue. The mammalian heart was the only material in which glutamine regularly constituted the major part. In all other tissues examined including avian heart, glutamic acid usually predominated, though in varying degrees. In the spleen the ratio glutamic acid/glutamine was 6–10, in brain about 2, in most other tissues variable.

In foetal tissues (Table 2) the sum of glutamic acid and glutamine was generally lower than in the adult tissues, an exception being the lung. The ratio glutamic acid/glutamine was higher in most foetal tissues than in the adult tissue, especially in the heart. Among the foetal tissues thymus showed the highest concentration of glutamic acid.

Human blood plasma Fifty-four specimens from fasting hospital patients were examined (Table 3). In all normal cases the average content of glutamic acid was 3.47 mg/100 ml and of glutamine 5.78 mg/100 ml. No major deviations were found in the forty-three pathological specimens. In the group 'infectious diseases', the glutamic acid values seemed to be higher, and the glutamine values lower than in the other groups, but in view of the small number of cases no definite conclusions can be drawn. As in other materials, the concentration of glutamic acid + glutamine showed much less variation than did the concentrations of the two components separately. The average sum of all cases was 8.74 mg/100 ml, of which 42.6% was glutamic acid and 57.4% glutamine. The data are in general

Table 1 *Glutamic acid, glutamine and ammonia in animal tissues*

Tissue	Animal	Amounts of substance found (10^{-4} mol/g)			
		Glutamic acid	Glutamine	Glutamic acid + glutamine	Ammonia
Liver	Sheep	5.45	2.27	7.72	2.68
	Sheep	6.53	0	6.53	2.08
	Sheep	5.47	2.66	8.13	1.21
	Cat	2.86	3.66	6.52	4.08
	Pigeon	6.46	0	6.46	2.68
	Pigeon	5.53	5.93	11.46	2.50
	Pigeon	5.75	5.35	11.10	6.15
Spleen	Sheep	10.60	1.47	12.07	3.39
	Sheep	9.59	1.56	11.15	3.16
	Sheep	10.95	1.01	11.96	3.18
	Cat	9.55	1.38	10.93	3.39
Kidney cortex	Sheep	7.90	1.21	9.11	2.16
	Sheep	4.86	0	4.86	4.24
	Sheep	5.95	0.87	6.82	4.17
	Cat	9.41	1.12	10.53	4.55
Kidney medulla	Sheep	7.00	1.79	8.79	1.96
	Sheep	4.23	2.08	6.31	1.16
	Sheep	7.08	0	7.08	4.59
	Cat	5.30	3.70	9.00	4.19
Brain, grey matter	Sheep	11.1	4.19	15.29	2.71
	Sheep	9.93	3.36	13.29	4.39
Brain, white matter	Sheep	7.01	3.36	10.37	1.56
	Sheep	5.30	3.28	8.58	2.88
Brain, whole	Cat	9.93	5.27	15.20	2.05
	Pigeon	6.16	6.99	13.15	0.93
	Pigeon	13.95	4.52	18.47	5.71
	Pigeon	8.25	5.75	14.00	2.88
	Pigeon	10.28	5.60	15.88	6.06
Lung	Sheep	2.38	1.54	3.92	2.08
	Sheep	3.67	0	2.67	3.74
	Sheep	4.15	0.73	4.88	0.94
	Cat	5.17	1.85	7.02	6.37
	Pigeon	1.67	2.14	3.81	4.08
	Pigeon	3.19	0.80	3.99	4.77
Heart	Sheep	2.99	14.0	16.99	1.79
	Sheep	2.09	11.2	13.29	1.87
	Sheep	1.34	9.25	10.59	1.74
	Cat	5.17	9.89	15.06	8.37
	Pigeon	3.13	5.41	8.54	4.95
	Pigeon	7.80	2.05	9.85	2.08
	Pigeon	6.34	3.64	9.98	6.25
Pancreas	Sheep	4.87	1.94	6.81	2.05
	Sheep	3.01	0	3.01	3.14
	Sheep	5.66	2.41	8.07	3.39
	Pigeon	5.53	7.54	13.07	7.83
	Pigeon	9.56	3.14	12.70	7.20
Skeletal muscle	Sheep	5.58	2.41	7.99	2.50
	Sheep	6.73	7.08	13.81	2.08
	Cat	0.74	5.55	6.29	6.37
	Pigeon	0	3.28	3.28	7.70
	Pigeon	8.15	1.03	9.18	5.08
	Pigeon	3.30	2.48	5.78	6.81
Smooth muscle, gizzard	Pigeon	10.52	3.56	14.08	2.72
	Pigeon	6.69	1.78	8.47	2.61
	Pigeon	2.72	3.90	6.62	4.91
Testis	Sheep	4.28	4.48	8.76	2.48
	Sheep	7.82	2.14	9.96	3.92

Table 1 (cont)

Tissue	Animal	Amounts of substance found (10^{-6} mol/g)			
		Glutamic acid	Glutamine	Glutamic acid + glutamine	Ammonia
Ovary	Sheep	1.32	1.63	2.95	1.36
	Sheep	4.21	0.58	4.79	1.94
Suprarenal gland (mainly cortex)	Sheep	3.55	1.52	5.07	4.91
	Cow	4.35	0.74	5.09	1.96
Thyroid gland	Sheep	0.80	0.47	1.27	3.52
	Cow	2.28	0	2.28	1.61
	Cow	4.28	0	4.28	2.68
Lymph gland	Sheep	6.09	2.28	8.37	4.35
	Cow	9.18	2.27	11.45	3.01
	Cow	6.60	1.65	8.25	0.40
Gastric mucosa	Sheep	2.70	0.94	3.64	3.18
Duodenal mucosa	Sheep	5.44	1.21	6.65	5.42
	Cat	5.03	3.60	8.63	6.34
Vitreous humour	Sheep	0.87	0	0.87	1.36
Lens	Sheep	1.07	0.58	1.65	0.67
Fat, peritoneal	Sheep	0.62	0.45	1.07	1.23

Table 2 *Glutamic acid, glutamine and ammonia in foetal tissues and placenta*

Tissue	Animal	Approx age of foetus (weeks)	Amounts of substance found (10^{-6} mol/g)			
			Glutamic acid	Glutamine	Glutamic acid + glutamine	Ammonia
Liver	Calf	10	3.78	1.03	4.81	1.79
	Calf	30	2.74	3.90	6.64	1.47
	Sheep	6-7	3.68	1.87	5.55	4.42
Brain (whole)	Calf	10	4.76	1.05	5.81	1.56
	Calf	30	8.16	2.61	10.77	2.07
Brain	Sheep	6-7	2.34	2.28	4.62	1.87
Kidney	Calf	10	5.55	0	5.55	1.47
	Calf	30	4.82	0.62	5.44	2.03
Heart	Calf	10	7.85	2.49	10.34	1.16
	Calf	30	4.02	6.36	10.38	2.54
	Sheep	6-7	0.49	0	0.49	5.90
Lung	Calf	10	7.28	0.89	8.17	0.94
Thymus	Calf	10	9.85	0.94	10.79	5.34
	Calf	30	11.30	0.89	12.19	2.81
Placenta	Calf	10	7.30	1.92	9.22	1.63
	Calf	30	6.76	2.03	8.79	1.94
	Sheep	6-7	2.45	2.05	4.50	4.86
	Cat	Almost full term	2.54	5.17	7.71	1.87
Spleen	Calf	30	6.96	0.60	7.56	2.52
Bone marrow, femur	Calf	30	2.01	1.74	3.75	2.94

agreement with the glutamine determinations in blood plasma published by Harris (1943), Archibald (1944) and Prescott & Waelsch (1947), though the present average values are a little lower. The average values for glutamic acid, in contrast, are somewhat higher than those reported by Prescott & Waelsch

DISCUSSION

Glutamic acid and glutamine in animal tissues The literature does not contain many data which are comparable with those presented in this paper. Ferdman *et al* (1942) examined the readily hydrolyzable 'amide nitrogen' in various tissues of the dog, cat, rabbit, pigeon and horse, the fresh tissue was frozen in liquid air, extracted with trichloroacetic acid and the increase of ammonia formed on hydrolysis (5–10 min, 100°, 5% sulphuric acid) was determined. Hamilton (1945) heated the picric acid extract of dog tissues to 100° at pH 6.5 for 90 min, and estimated the decrease in 'carboxyl nitrogen' by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941). The figures obtained by Ferdman *et al* (1942) and Hamilton (1945) are of the same order as those reported in the present paper.

Table 3 *Glutamine and glutamic acid in human blood plasma*

(The results are expressed, in accordance with the practice of previous authors, as mg/100 ml plasma. Glutamine is expressed as glutamic acid.)

	Plasma (mg/100 ml)		
	Range	Mean	s.d.
Normal (11 cases)			
Glutamic acid	1.3–5.9	3.41	1.39
Glutamine	2.7–7.8	5.78	1.55
Total	7.6–10.1	9.19*	0.84
Diseases of the circulatory system (13 cases)			
Glutamic acid	1.7–5.3	3.74	1.13
Glutamine	3.5–8.0	5.60	1.18
Total	6.2–10.6	9.34	1.17
Malignant tumours (5 cases)			
Glutamic acid	1.4–4.6	2.94	1.12
Glutamine	2.6–7.6	5.02	2.08
Total	7.2–9.8	7.96	1.07
Infectious diseases (12 cases)			
Glutamic acid	2.4–6.6	4.17	1.42
Glutamine	2.0–6.8	4.05	1.58
Total	6.3–10.2	8.22	1.27
Miscellaneous (13 cases)			
Glutamic acid	0*–7.2	3.96	2.14
Glutamine	2.0–9.2	4.64	2.27
Total	5.6–11.4	8.60	2.11

* No glutamine was present in one case of thyrotoxicosis (basal metabolic rate +29%).

Hamilton (1945) has already pointed out that in cardiac muscle of the dog glutamine contributes 50–60% of the free total 'carboxyl nitrogen' of the tissue. A comparison of the present data with estimations of the total amino nitrogen in animal tissues (Van Slyke, 1913, Hamilton, 1945) indicates that in most tissues the sum of glutamic acid and glutamine represents 25–60% of the total amino nitrogen.

Van Slyke (1913) was the first to note that tissues contain 5–10 times more amino nitrogen than blood plasma. Hamilton found a similar proportion for glutamine. The present data show that this is also true for glutamic acid.

The question may be raised whether the glutamic acid found in tissue suspension has, wholly or in part, arisen from glutamine after death, as a result of the action of glutaminase. The ammonia values given in Tables 1 and 2 are in most tissues much lower than the glutamic acid values. This applies especially to the tissues which are known to contain a glutaminase (liver, kidney, brain). Most of the glutamic acid found in these tissues must, therefore, have been preformed.

Ammonia in animal tissues Many data are available on the ammonia content of blood and of other body fluids, but of the tissues only cardiac and striated muscle have been thoroughly studied, mainly by the schools of Parnas and Embden. As Parnas (1928) has pointed out, it is uncertain whether ammonia found in animal tissues, even in material treated with liquid air, is preformed or arises after death. This has to be borne in mind in the interpretation of results.

SUMMARY

1 The decarboxylase method (Gale, 1945, Krebs, 1948) has been used to determine separately glutamic acid and glutamine in twenty-four different animal tissues (including foetal material), and in fifty-four specimens of human blood plasma.

2 The sum of the concentrations of glutamic acid and glutamine was highest in brain cortex, heart, spleen and thymus (average $10\text{--}15 \times 10^{-6}$ mol/g). Average values below 5×10^{-6} mol/g were found in ovary, thyroid and lung, and below 1×10^{-6} mol/g in most other tissues.

3 In the mammalian heart and in blood plasma glutamine was present in greater quantities than glutamic acid. In other tissues glutamic acid predominated as a rule.

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A Method for Determining the Sedimentation Constant of Material of Low Molecular Weight: Studies on Oxidation Products of Insulin

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In a recent paper one of us (Gutfreund, 1948) has shown that insulin molecules probably consist of sub-units of molecular weight 12,000, these units polymerize to molecules of weight 48,000 in neutral solutions of moderate insulin concentration (0.5–1%). The mean molecular weight is, however, dependent upon pH, temperature and concentration. From the determination of terminal amino groups of insulin, Sanger (1945) suggested that these sub-molecules, of molecular weight 12,000, are made up of four peptide chains bound together by disulphide linkages. Two of these chains have terminal glycyl residues and two have terminal phenylalanyl residues. Sanger (1947) has shown that the disulphide linkages can be split by oxidation with performic acid, without affecting any other part of the insulin molecule. Some preliminary studies on the peptides resulting from this oxidation of insulin have been reported by Sanger (1947).

It was the purpose of the work described in this paper to develop methods for the determination of sedimentation constants (S_{20}) less than 1×10^{-13} and to study fractions from oxidized insulin both by ultracentrifugal sedimentation and by diffusion. Sanger (1947) suggested that the peptides obtained on oxidation of insulin should have a molecular weight of about 3000. This value is between the ranges of molecular weights which have been studied by methods suitable for macromolecules (osmotic pressure, sedimentation and diffusion, etc.) on the

one hand, and those used for simpler compounds (freezing-point depression and similar procedures) on the other hand. It was necessary to modify the procedure of computing sedimentation constants to make it useful for the purpose of studying these polypeptides.

EXPERIMENTAL

Ultracentrifugal examinations were carried out in a Svedberg oil turbine ultracentrifuge, and the Philpot (1938) optical system was used for the observation of the boundaries. The speed of the centrifuge was about 1010 r.p.m. Diffusion constants were determined by the method of Coulson, Cox, Ogston & Philpot (1948).

Dr F. Sanger kindly prepared for us all the oxidized insulin and fractions thereof used in this work. The material was prepared and fractionated as described by Sanger (1949). Crystalline zinc insulin (obtained from Boots Pure Drug Co. Ltd.) was used as starting material. Two fractions (A and B) were examined. Fraction A contains the peptides with terminal glycyl residues while fraction B contains those with terminal phenylalanyl residues. The purity of each fraction, as shown by end group assay, was about 95%.

It was found that oxidized insulin diffused slowly through collodion or cellophan membranes on dialysis, as it was essential to get the solutions of oxidized insulin into equilibrium with a salt solution of known composition, these were dialyzed against a large volume of $M Na_2HPO_4$. Up to half the nitrogenous material was lost from the solutions.

Method of calculation of the sedimentation constant in the absence of a clear boundary of sedimentation

Two methods are in general use for the observation of sedimentation constants of proteins, first the light absorption method, giving a direct plot of concentration c against distance x in the cell, and secondly the refractive index method, giving a plot of concentration gradient dc/dx against x . Whichever method is used, the sedimentation constant is normally calculated from the movement of the mid-point of the protein boundary with time. This procedure is accurate only if the calculations are made from measurements on boundaries which have completely left the meniscus of the solution and have not reached the bottom of the cell. The peptides studied during this investigation have such small sedimentation and high diffusion constants that one end of the boundary is at the bottom of the cell by the time the mid-point (or peak) has left the meniscus, and it is, therefore, impossible to get successive pictures of the whole boundary (see Fig 1)

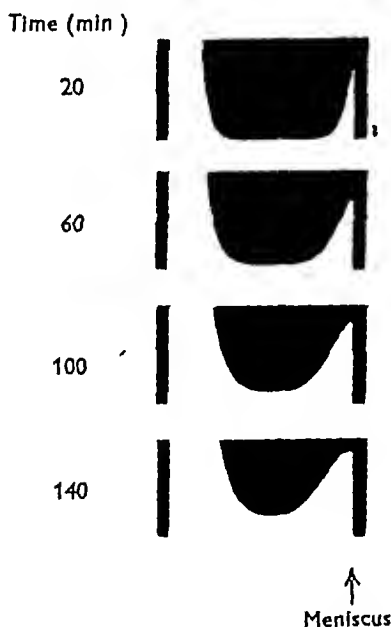


Fig 1 Philpot schlieren diagrams of fraction A 20, 60, 100 and 140 min after full speed had been attained. Oxidized insulin concentration 0.74%

It has been suggested by Tiselius, Pedersen & Svedberg (1937) that, in such cases, the movement of the quantity of protein across an arbitrary plane in the cell could be used for the calculation of the sedimentation constant. This method was elaborated by Svedberg & Pedersen (1940), but no practical use appears to have been made of it so far, and no working procedure has been described, and we have, therefore, developed the following method for the

calculation of the sedimentation constant of very slowly sedimenting substances from photographs obtained by the Philpot (1938) optical method.

Fig 2 is a diagram of the ultracentrifuge cell, with a plot of the concentration and of the gradient of concentration. A fixed plane, distant X cm from the centre of rotation, is chosen so that the concentration in its neighbourhood varies only with time t .

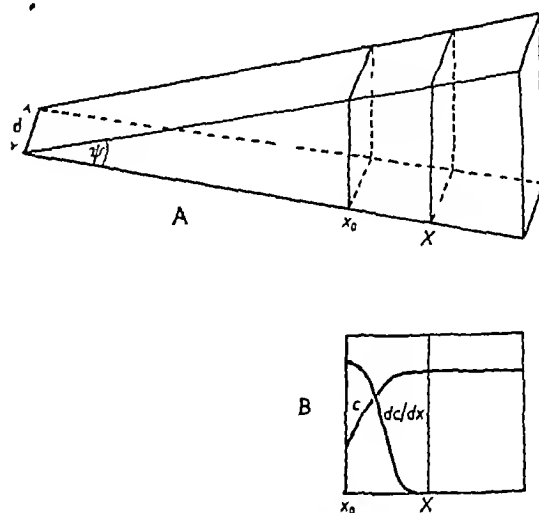


Fig 2 A, diagram of the ultracentrifuge cell showing the dimensions used in the calculation, B, plots of c and dc/dx against x

and not with the distance x from the centre of rotation. The sedimentation constant can be obtained from the rate at which material passes the plane X . The concentration at X (and throughout the cell) is initially c_0 , but after time t (assuming that the speed of rotation ω and the sedimentation constant s do not vary) it has fallen to

$$c_{xt} = c_0 e^{-2s\omega^2 t} \quad (1)$$

The rate at which material is passing X at time t is, therefore,

$$\begin{aligned} \frac{dQ}{dt} &= c_{xt} s \omega^2 X \psi X d \quad dt \\ &= \psi d c_0 s \omega^2 X^2 e^{-2s\omega^2 t} dt \end{aligned}$$

Integrating between 0 and t , the amount of material which has passed across X from the upper region of the cell is

$$\Delta Q_t = \psi d \frac{c_0 X^2}{2} (1 - e^{-2s\omega^2 t}) \quad (2)$$

This quantity ΔQ_t can be obtained from the schlieren diagrams. The concentration at x , t is given by

$$c_{xt} = c_{xt} - \int_x^X \frac{dc}{dx} dx \quad (3)$$

The quantity of material above X at time t is then given by

$$Q_t = \int_{x_0}^X c_{x,t} \psi x dx$$

which, from (1) and (3),

$$\begin{aligned} &= \psi d \left\{ \int_{x_0}^X c_0 e^{-2s\omega^2 t} x dx - \int_{x_0}^X x \int_x^X \frac{dc}{dx} dx dx \right\} \\ &= \psi d \left\{ c_0 (X^2 - x_0^2) e^{-2s\omega^2 t} / 2 - \int_{x_0}^X x \int_x^X \frac{dc}{dx} dx dx \right\}, \quad (4) \end{aligned}$$

The quantity initially above X was

$$Q_0 = \int_{x_0}^X c_0 \psi x dx = \psi d c_0 (X^2 - x_0^2) / 2 \quad (5)$$

Since $\Delta Q_t = Q_0 - Q_t$ and writing Q'_t for the second term in the bracket of (4),

$$c_0 X^2 (1 - e^{-2s\omega^2 t}) / 2 = c_0 (X^2 - x_0^2) / 2 - c_0 (X^2 - x_0^2) e^{-2s\omega^2 t} / 2 + Q'_t,$$

whence $Q'_t = c_0 x_0^2 (1 - e^{-2s\omega^2 t}) / 2$

This expression is exact. It can be solved, without further approximation, by converting it into the form

$$\log \left\{ 1 - \frac{2Q'_t}{c_0 x_0^2} \right\} = -\frac{2s\omega^2}{2 \cdot 303} t \quad (6a)$$

By plotting the log term against t , a straight line of slope $-2s\omega^2/2 \cdot 303$ should be obtained. Where $s\omega^2 t$ is small, equation (6a) approximates to

$$Q'_t = c_0 x_0^2 s \omega^2 t \quad (6b)$$

This method of solution amounts to the assessment of the amount of material which would have passed the plane at x_0 had the solution not been terminated there by the meniscus, this is measured by the amount of material which is absent below x_0 compared with expectation, making allowance for the 'dilution factor'.

Computation

Enlarged projections ($\times 4.5$) of the boundary photographs were traced on to graph paper, the base lines were fitted from the corresponding photographs of a control run done on buffer alone. Values of $\int_x^X \frac{dc}{dx} dx$ were obtained for a series of values of x in each tracing by counting squares, and were converted into units of refractive increment by use

of the appropriate optical constants. Each value was then multiplied by the corresponding value of x (the actual distance from the centre of rotation) and each product $x \int_x^X \frac{dc}{dx} dx$ was plotted against x . Graphical integration of each resulting curve between x_0 and X gave the value of Q'_t .

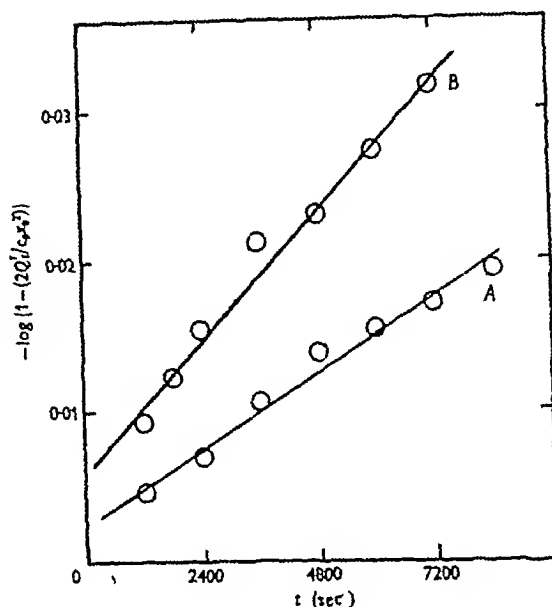


Fig 3 Plot of $-\log \left\{ 1 - \frac{2Q'_t}{c_0 x_0^2} \right\}$ (in terms of refractive index) against time of sedimentation t from reaching full speed, for fractions A and B

Fig 3 shows the values of $-\log (1 - (2Q'_t / c_0 x_0^2))$ plotted against t for the two fractions of oxidized insulin. The variations of ω^2 and of the temperature together affect the rate of sedimentation by less than 1% and their mean values have therefore been used, the regression coefficient b and the standard deviation of b have been calculated in each case. The sedimentation constant s is then given by

$$s = 2 \cdot 303 b / 2\omega^2$$

The sedimentation constant in water at 20° , $S_{20}(\text{corr})$, is obtained by applying the usual corrections to s , including that for error of reading of the cell temperature (Cecil & Ogston, 1949).

RESULTS

The values of $S_{20}(\text{corr})$ and of $D_{20}(\text{corr})$ (measured at the same concentrations) are given in Table 1, the molecular weights have been obtained from these values, assuming a value of 0.75 for the partial specific volume.

Table 1 *Sedimentation and diffusion constants of oxidized insulin fractions*

	$S_{20}(\text{corr})$ $\times 10^{13}$	S_D of $S_{20}(\text{corr})$ $\times 10^{13}$	$D_{20}(\text{corr})$ $\times 10^7$	Molecular wt
Fraction A	0.53	0.034	17.6	2900
Fraction B	0.91	0.054	12.6	7000

DISCUSSION

The diffusion results indicated that fraction A was nearly homogeneous, whereas fraction B was heterogeneous. The method of preparation would also suggest that fraction A is the more homogeneous, fraction B probably containing a certain amount of incompletely oxidized insulin.

The simplest assumption from Sanger's (1945) data is that the unit of insulin is split by oxidation into four peptide chains, two of these having glycyl end-groups (fraction A) and the other two having phenylalanyl end-groups (fraction B). The molecular weight estimated for the former agrees moderately with Sanger's (1947) estimate of 2500 from analytical data. By difference from the total weight of 12,000, the molecular weight of fraction B should then be about 3100, the discrepancy between this and the value estimated suggests that fraction B contains a considerable proportion of incompletely

oxidized material, which would seriously affect the values of both sedimentation and diffusion constants.

The main purpose of this paper was to describe a general method for studying these polypeptides in the ultracentrifuge, the results of the examination of the oxidation products of insulin are necessarily only preliminary. It is possible that some fraction of much smaller molecular weight was lost during dialysis and the assumption of two chains with glycyl end groups and two with phenylalanyl end groups, making up the unit of molecular weight 12,000, may well be an oversimplification. The increase in molecular weight on oxidation (600 per 12,000) has not been taken into consideration in the above calculation.

SUMMARY

1 Details are given of a method of obtaining the sedimentation constant of material of too small a molecular weight to form a clear sedimenting boundary in the ultracentrifuge.

2 This method has been applied to the study of the peptides obtained by partial degradation of insulin with performic acid. The diffusion constants of two fractions have also been measured and estimates made of their molecular weights.

One of us (H G) is indebted to Prof R A Peters, F R S, for hospitality in his laboratory.

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Protease Inhibitors

1 ASSAY AND NATURE OF SERUM ANTIPROTEASE

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The inhibition of trypsin by normal serum has been studied by many workers (Landsteiner, 1900, Fujimoto, 1918, Hussey & Northrop, 1922-3, Grob, 1943). The association of the trypsin inhibitor with the albumin fraction first noted by Landsteiner (1900) has been confirmed by subsequent workers using salt fractionation, and Smith & Lindsley (1939) have shown that on electrophoretic separation of the serum proteins the antitryptic power lay mostly in the albumin fraction. Several groups of workers (Schwartz, 1909, Bauer, 1910, Jobling & Petersen, 1914) have associated antitryptic activity in serum with lipids or fatty acids, and Ungar (1945) has found that it was removed by ether extraction and reconstituted by readdition of the ether extract to the aqueous residue. This association of antitryptic activity with lipids has been denied by many others (Meyer, 1909, Coblner, 1910, Teale & Bach, 1919-20).

Studies by Christensen & MacLeod (1945) and MacFarlane & Pilling (1946) show that plasma trypsin (plasmin) is likewise inhibited by a constituent of the albumin fraction of plasma which the latter authors term antiplasmin. Beloff (1946) has shown that the skin protease studied by Beloff & Peters (1944, 1945, 1946) is also inhibited by the albumin fraction of serum. Her work clearly demonstrates that the inhibitory factor against skin protease is identical with the inhibiting factor for both trypsin and plasma trypsin, although she drew the opposite conclusion from a study of the literature. Schmitz (1937, 1938) claims to have isolated from serum a second antitryptic factor which he regards as a polypeptide similar in properties to that isolated from pancreas by Kunitz & Northrop (1936), and which he believes is normally present in combination with plasma trypsin, thereby inactivating it. The evidence brought forward by Schmitz is hardly sufficient to support his claims. Grob (1943) appears to have prepared a 'crystalline' serum antitrypsin by the methods of Schmitz. Many authors have described marked physiological and pathological variations in the antitrypsin activity of the sera of man and animals and a good summary is given by Grob (1943). The majority of the conditions reported showed a marked increase over the normal level, but no single factor appears to be

responsible, and the work is difficult to assess because of the wide variation in methods used, and the lack of any simple accurate technique for assaying serum antitrypsin.

The development by Swyer & Emmens (1947) of a satisfactory viscosimetric relationship (in the case of hyaluronidase) between enzyme activity and flow time, as measured in Ostwald viscosimeters, made it possible to assay serum antitrypsin viscosimetrically using gelatin as substrate and to restudy some of the findings of previous workers.

EXPERIMENTAL

Materials and methods

Gelatin solution Gelatin 10% (w/v) in 0.85% (w/v) NaCl (saline) was autoclaved and stored in the cold in 100 ml amounts. After warming, 10 ml of 0.2M phosphate buffer (pH 7.6) were added to every 90 ml gelatin, and the mixture was then brought to pH 8.0 by addition of a few drops of 2N NaOH. The solution was heated to nearly 100° and filtered through a Whatman no. 1 filter paper, the pH was adjusted to 7.6, and saline added so that the final relative viscosity after dilution with an equal volume of 0.5% (w/v) gum acacia in saline was about 2.3. The gelatin was stored in the cold until next day when it was melted at 37.5° and kept at that temperature during use. Merthiolate was added as a preservative.

Trypsin solution Crystalline trypsin containing approximately 50% (w/v) MgSO_4 was dissolved at 0.1% (w/v) concentration in 0.01N HCl and stored at 4°. Dilutions were made in 0.5% (w/v) gum acacia. As a standard of comparison, the enzyme was assigned the arbitrary value of 20,000 units/mg.

Estimation of enzyme and inhibitor Equal volumes of enzyme and gelatin solution were mixed at 37°, and 2 ml of the mixture transferred to Ostwald viscosimeters in a water bath at 37.5°. The viscosity of the mixture was measured 20 min after mixing, and this was carried out more accurately by taking readings at 16, 20 and 24 min respectively and interpolating when necessary. Mixtures of inhibitor and enzyme were incubated for 10 min at 37° prior to mixing with gelatin. The viscosimeters used had a bulb capacity of a little more than 1 ml, a capillary of 0.5 mm diam and a flow time of 51 ± 3 sec.

Viscosimetric assay of enzyme and inhibitor

Enzyme Although several investigators (Hussey & Northrop, 1922-3, Northrop & Kunitz, 1932-3, Smith & Lindsley, 1939, Christensen, 1945) have used viscosimetric

methods for the assay of proteolytic enzymes using gelatin as a substrate, it is doubtful if any of the formulae developed, which relate enzyme concentration to a rate of fall in viscosity, are entirely satisfactory. Swyer & Emmens (1947), in a study of hyaluronidase action, defined the flow-time index v as

$$\frac{(f_s - f_o)}{(f_s - f_o)} \times 1000,$$

where f_s = flow time of substrate and enzyme solution, f_o = flow time of solvents alone (these being in the same proportion as in the hydrolysis mixture), and f_e = the flow time of the enzyme substrate mixture after incubation for a given time such as 20 min. They showed that v gives a straight line relationship when plotted against the logarithm of the enzyme concentration.

Since $f_s - f_o$ is the viscosity due to the substrate, and $f_s - f_e$ represents the reduction of viscosity caused by the action of the enzyme on the substrate, and since the curve relating flow time to substrate concentration is approximately linear at the concentrations of hyaluronic acid used,

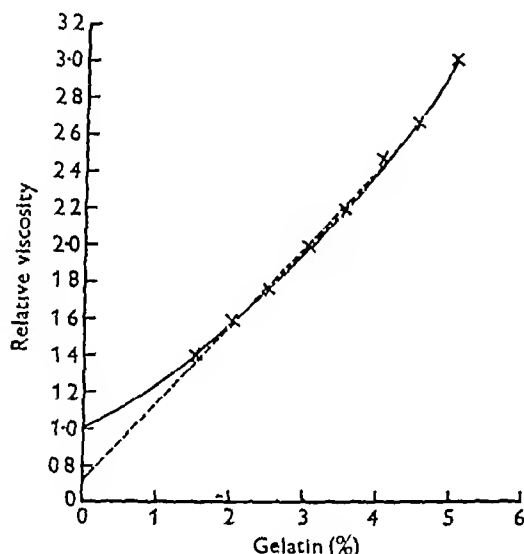


Fig 1 Relationship between concentration of gelatin and relative viscosity as determined in an Ostwald viscometer

their finding evidently rests on the assumption that the flow time index v is a measure of the fraction of the total substrate hydrolyzed in a given time. Between relative viscosities 2.7 and 1.6 the curve relating relative viscosity (or flow time) to gelatin concentration is likewise approximately linear (Fig 1). The line does not pass through viscosity 1.0 as it would if a lower range of concentration were considered, but through a value, in this case 0.76, which depended on the batch of gelatin used and showed little tendency to vary. Working between initial and final relative viscosities of 2.5 and 1.6, an approximately linear relationship (Fig 2) was found when v , the flow time index, was plotted against the logarithm of the enzyme concentration for values of v between 150 and 730. The flow-time index can, therefore, be used as a measure of the amount of substrate hydrolyzed within a given range of viscosities, provided that these viscosities give a straight line when plotted against gelatin concentration and irrespective of where the line cuts the ordinate.

Gelatin as used in the assays increased slightly in viscosity during the first 3 hr incubation at 37.5°, after which it underwent a slow loss amounting to between 0.01 and 0.02 unit relative viscosity/hr. Neither this slight fall nor the day to day variations encountered in making up the solutions had any significant effect on the value of v obtained provided the f_s values were redetermined at intervals.

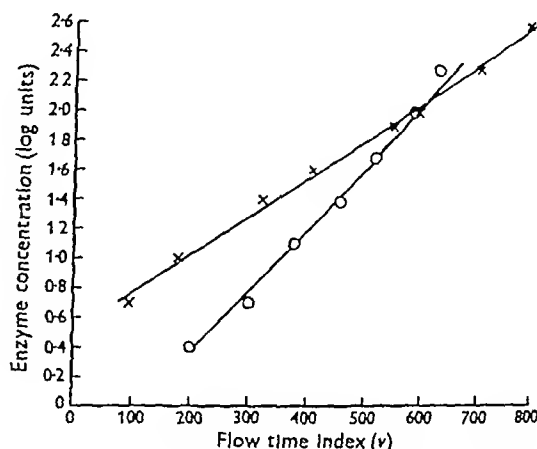


Fig 2 Relationship between flow time index (v) and logarithm of enzyme concentration for crystalline trypsin and crystalline chymotrypsin. \times — \times Crystalline trypsin, \circ — \circ crystalline chymotrypsin.

Table 1 shows that even large variations in the initial viscosity of the gelatin from 2.5 to 1.96 altered v by only 40, there being a rise of about 7.4 in the flow time index for each fall of 0.1 in the relative viscosity of the gelatin. In practice it was sufficient to adjust the viscosity each day to the same value, and to redetermine it at intervals on one

Table 1 Effect of change in initial viscosity of gelatin on the flow time index v obtained with crystalline trypsin: each value an average of four to six determinations

Relative viscosity	v	
	Trypsin (100 units)	Trypsin (25 units)
2.50	563	263
2.20	590	288
1.96	604	304

viscosimeter, since the slope of the line relating the logarithm of the enzyme concentration to the flow time index was identical at different viscosities, and for many months the flow-time index for 100 units of crystalline trypsin was constant at about 600. Slightly different values were obtained with other samples of gelatin. The slope of the line obtained with crude trypsin was identical, but that of crystalline chymotrypsin (Fig 2) was different.

RESULTS

Rate of reaction of the inhibitor on enzyme. The inhibition of crystalline trypsin by serum increases with the time of contact before the addition of

substrate (Fig 3), the main part of the reaction being completed in the first 20 min. The 10 min incubation period used in the course of the work was based on preliminary trials with crude trypsin, which showed very little increase in inhibition by serum after this time.

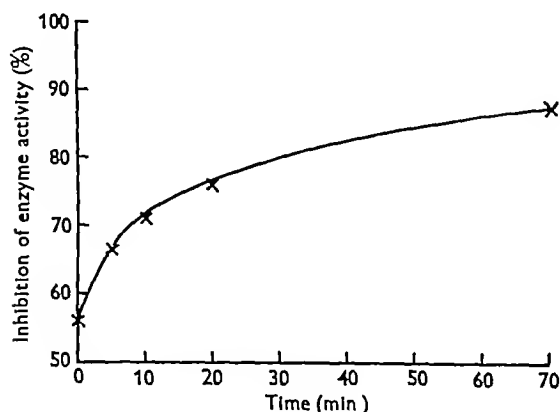


Fig 3 Effect of time of contact of serum and trypsin solution at 37° before mixing with gelatin on the inhibition of enzyme

Effect of concentration of inhibitor on degree of inhibition The inhibitory action of varying concentrations of serum on given concentrations of crude and crystalline trypsin of equal activity is shown in Fig 4, where the residual activity as

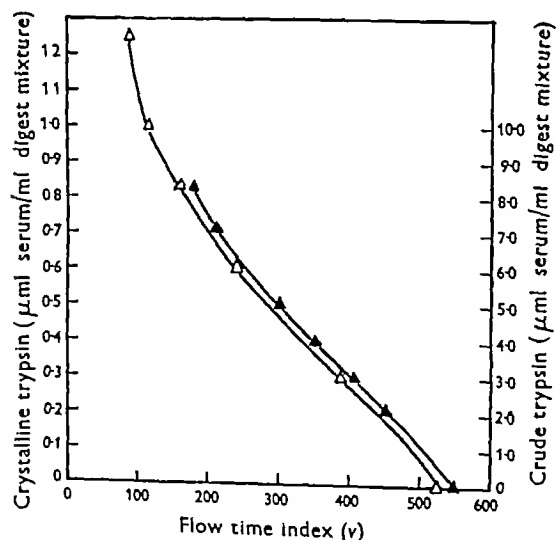


Fig 4 Effect of addition of varying amounts of serum on flow time index of 75 units/ml of crude and crystalline trypsin (1 mg crystalline trypsin = 20,000 units) Δ — Δ , crystalline trypsin + guinea pig serum, \bullet — \bullet , crude trypsin + horse serum

measured by v is plotted against volume of serum added. Apart from the fact that some ten times more serum was required to produce a comparable degree of inhibition in the case of crude trypsin, the

curves are identical. When the percentage inhibition of enzyme activity at each serum level is calculated by a comparison of Figs 2 and 4, and this is plotted against the serum volumes added, a curve is obtained (Fig 5) which is very similar to that obtained by

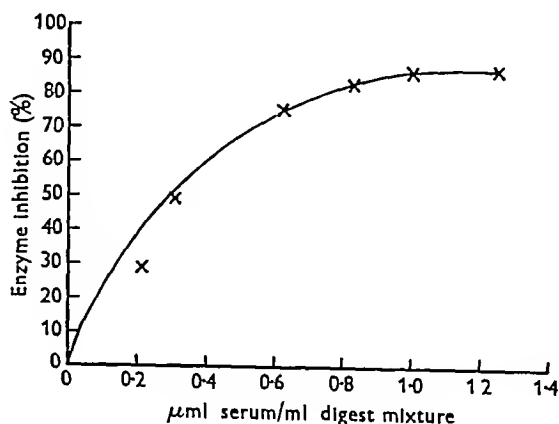


Fig 5 Effect of increasing amounts of serum on a constant amount of enzyme (75 units/ml) expressed as per cent enzyme inhibition (1 mg crystalline trypsin = 20,000 units)

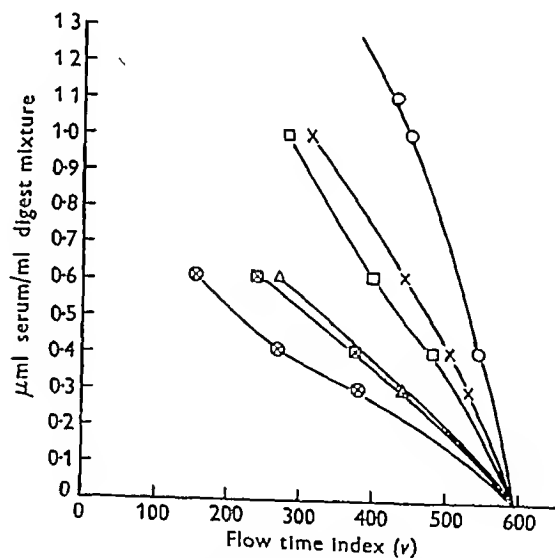


Fig 6 Inhibition of trypsin by various sera as measured by the flow time index, O, rabbit, □, horse, Δ, sheep, x, normal human, ⊗, human (pregnancy), ⊙, human (tuberculosis)

Hussey & Northrop (1922-3), who studied the action of serum on crude trypsin, and of Kunitz (1947), who investigated the effect of soya-bean trypsin inhibitor on crystalline chymotrypsin. Kunitz has derived an equation, relating the degree of inhibition and the amount of soya-bean inhibitor added to a constant amount of trypsin, on the basis of this being a uni-molecular reversible reaction. The curve in Fig 5 is similar to that derived from Kunitz's equation though the agreement was not exact.

A comparison of the inhibitory power of various sera on trypsin can be made by reference to the curves obtained when various amounts of serum were added to a given amount of enzyme, such as crystalline trypsin (Fig 6). A comparison of the volumes of each serum (measured on the curves) required to produce the same degree of inhibition shows good agreement when measured at $v=500$ and $v=400$ (Table 2). Since eight samples of normal

Effect of fat solvents The most marked destruction of the inhibitor was obtained by shaking and incubation with chloroform which acts as a protein denaturant (Table 4). Several attempts were made to repeat the findings of Ungar (1945), who claims that, whilst after ether extraction both the aqueous and ether fractions of normal guinea pig serum have slight antitryptic action, their antitryptic power on recombination is equal to that of the original serum

Table 2 Comparison of inhibitory power of various sera against crystalline trypsin for values of v in Fig 6

Serum	$v=500$		$v=400$	
	Serum (ml)	Volumes containing same activity relative to rabbit serum = 100	Serum (ml)	Volumes containing same activity relative to rabbit serum = 100
Rabbit	0.66	100.0	1.22	100.0
Human	0.42	63.5	0.72	59.0
Horse	0.35	53.0	0.60	49.0
Sheep	0.20	30.0	0.40	32.8
Human (pregnancy)	0.19	28.5	0.37	30.0
Human (tuberculosis)	0.13	19.9	0.25	20.5

human serum taken on two successive days from four persons showed little or no difference in inhibitory power when compared in this manner, fresh normal human serum was used as the basis of comparisons carried out at different periods during the course of the work. Similar lack of variations in inhibitory power within individual species was encountered in the case of other normal animal sera.

Characteristics of the normal serum trypsin inhibitor

Stability of serum trypsin inhibitor The inhibitor was more unstable in the acid than in the alkaline range and there was some species variation (Table 3).

Table 3 Stability of serum trypsin inhibitor under various conditions

(In all experiments except the last, the serum was first diluted to 10% (v/v) in saline.)

Time	Temp (°)	pH	Loss in inhibitory power (%)	
			Sheep	Horse
2 hr	37	4.6	27	80
2 hr	37	9.6	Nil	Nil
30 min	60	7.4	20	46
30 min	70	7.4	33	70
30 min	80	7.4	95	70
12 months	4	—	Nil	Nil

There was a considerable loss above 60° in both horse and sheep sera. The loss on storage at 4° in both sera was very small, and there was even evidence of some increase.

No details are given by Ungar, but in the present studies neither prolonged shaking nor reflux distillation for 20 min of both guinea pig and horse sera with ether removed more than a small fraction (often none) of the total activity, and this slight loss could

Table 4 Stability of sheep serum inhibitor on exposure to fat solvents

(10% (v/v) solvent added, shaken 10 min and incubated 5 hr at 37°, activity measured at final serum dilution 1/1600 (v/v) against 100 units crystalline trypsin.)

	v	Loss in inhibitory power (%)
Trypsin alone	575	—
Trypsin + serum treated with saline	163	—
" " " " acetone	290	32
" " " " ethanol	188	Nil
" " " " ether	157	Nil
" " " " chloroform	476	75

not be restored by readdition of the extract. Ether extraction at -20° resulted in a 20% loss which was not restored by readdition of the ether soluble fraction. The inhibitor could not be extracted by acetone or chloroform.

Precipitation by trichloroacetic acid and dialysis The antitryptic factor was precipitated with a loss of about 50% by the addition of an equal volume of 5% (w/v) trichloroacetic acid to sheep serum. The supernatant fluid was without activity. Sera diluted 1/10 and brought to various pH values were pressure dialyzed through cellophane membranes, but the filtrates were without activity.

Salt fractionation Serum treated with ammonium sulphate showed a marked loss in inhibitory power unless it was first buffered with sodium glycerophosphate, 7 % (w/v) Under these conditions at least 90 % of the total activity of sheep serum could be recovered (Table 5) A lower final yield was

Serum antitrypsin factor of Schmitz Schmitz (1937, 1938) claimed to have separated an antitryptic factor from serum, which appeared to resemble a polypeptide, and which he believed served as a bound inactivator of the plasma trypsin The antitryptic factor was obtained in acetone extracts of human plasma and by fractional precipitation of ox blood with ammonium sulphate following the addition of 2 vol of 0.5 N sulphuric acid In the present study no inhibitory activity was found in acetone extracts of human plasma following Schmitz's directions The fractional precipitation of 1.5 l of ox blood gave approximately 180 mg of material, representing a total inhibitory activity of only some 0.02 % of the original 1.5 l Two thirds of the product was ammonium sulphate, and this cannot be removed according to Schmitz since the inhibitor itself is dialyzable A similar product was obtained from human blood which was not destroyed by the addition of streptokinase as might have been expected from its supposed nature

Table 5 Comparison of inhibitory activity of serum fractions against crystalline trypsin after salting out

Fraction	Inhibitory activity as % of original serum		
	Albumin	Globulin	Total, reconstituted
Sheep serum once fractionated with (NH ₄) ₂ SO ₄	65	20	80
Horse serum once fractionated with Na ₂ SO ₄	32	13	65

obtained on fractionation by 18 % sodium sulphate for 1 hr at 37° using horse serum The inhibitor was present in both albumin and globulin fractions although there was a marked reduction in the inhibitory power of either fraction on subsequent precipitation Separated electrophoretically both fractions were inhibitory, the globulin being about twice as active on a weight basis

A similar fractionation to that made by Beloff (1946) with ammonium sulphate showed that about two thirds of the total inhibitory activity of the albumin fraction lay in the more soluble part (Table 6), that is the fraction precipitated by full

Variation in antitryptic level

(a) *Normal animals* Although the antitrypsin levels of different animal species varied considerably there was little or no variation between individual members of the same species

(b) *Anaphylaxis* Burdon (1942) claimed that there was a 100 % increase in the antitryptic power of the sera of guinea pigs sensitized with egg albumin which fell rapidly to normal following injection of a shocking dose None of these changes could be confirmed

Table 6 Comparison of inhibitory activity of various sheep serum protein fractions following ammonium sulphate precipitation

	Globulin	Total albumin (fraction A)	Albumin precipitated by 50 % saturation with (NH ₄) ₂ SO ₄ at pH 4.2 (fraction B)	Albumin precipitated by 100 % saturation with (NH ₄) ₂ SO ₄ at pH 4.2 (fraction C ₁)
			12	45
Activity recovered expressed as percentage total activity of serum	20	65		
Concentration of fraction in serum (%)	3.0	3.5	1.92	1.17
Activity/Concentration	6.6	18.5	6	38

saturation with ammonium sulphate at pH 4.2, and that on a weight basis this was about six times as active as either the less soluble fraction precipitated at pH 4.2 and 50 % (w/v) saturation or the globulin fraction A further precipitation of the more soluble fraction corresponding to Beloff's C₁ showed a loss of 50 % on a weight basis The figures given are very approximate, but serve to illustrate the wide difference in inhibitory power of the different fractions

(c) *Immunity* Grob (1943) claimed that the daily intramuscular injection of crude trypsin in rabbits over 4 weeks resulted in a rise of between 70 and 250 % in the antitrypsin titre of the animals, and that a similar, though less constant, rise was obtained in 4-8 days by the daily oral administration of the same product In the present studies there was a rise of some 15 % in the antitrypsin activity of the serum of a rabbit injected daily subcutaneously for 4 weeks with crude trypsin, but no

rise was observed following oral administration as claimed by Grob

(d) *Alterations in disease* Many observers have noted an altered level of serum antitrypsin activity in various pathological conditions in man and animals, usually associated with a considerable degree of cachexia. The sera of seven persons all showing marked cachexia were examined. An increase of about 150 % was found in the antitryptic titre of the serum of three cases of tuberculosis. The remaining four (two tubercular and two non-tubercular) were normal.

(e) *Pregnancy* The sera of eight women all in the terminal stages of pregnancy showed a uniform increase in the antitrypsin level of about 100 %.

DISCUSSION

The exact nature of the serum factor responsible for trypsin inhibition is still in doubt. Although associated with all fractions obtained by fractional salting out, it may yet be a single factor which is adsorbed on other proteins during precipitation. All attempts to separate it from albumin either by solvent extraction, adsorption or dialysis, under various conditions, have been unsuccessful, thus confirming the findings of other authors. Recently, Lineweaver & Murray (1947) have shown that the trypsin-inhibitory property of egg white is due to ovomucoid, and it is possible that the antitryptic action of serum is associated with a similar small molecular compound such as serum mucoid or seroglycoid (Hewitt, 1937). Unfortunately, the preparation of either of these substances, if they are indeed distinct (Meyer, 1938; Rimington, 1940), is carried out by methods likely to impair antitryptic properties, such as successive fractionation and crystallization at the isoelectric point (Hewitt, 1937), or by removal of the other proteins by boiling (Rimington, 1940). It will, therefore, be necessary to devise other methods for their isolation if this matter is to be tested.

A comparison of these studies with those of Beloff (1946) makes it clear that the serum inhibitor of skin protease and serum antitrypsin are identical. Serum trypsin is likewise inhibited by the same factor (Christensen, 1946). Beloff failed, however, to find any inhibitory activity against skin protease in the globulin fraction of serum, as did other authors who used pancreatic trypsin. In the present studies inhibitory activity was present in the globulin after two precipitations with ammonium or sodium sulphate, although it was much less than the activity after one precipitation. Globulin separated electrophoretically was about half as inhibitory on a weight basis as was albumin when measured against crystalline trypsin.

Finally there is no evidence that the inhibitor or portion of the inhibitor can be extracted with ether as claimed by Ungar (1945), nor is there any real evidence that serum contains a polypeptide inhibitor similar in its properties to the pancreatic trypsin inhibitor isolated by Kunitz & Northrop (1936), as is claimed by Schmitz (1937, 1938). The treatment of whole blood with 0.5 N sulphuric acid followed by fractionation between 40 and 70 % saturation with ammonium sulphate undoubtedly gives a product with weak inhibitory powers. The hypothesis that this product is a specific inactivator for plasma trypsin, similar to Northrop's inhibitor for pancreatic trypsin, is very much open to doubt, since it rests on nothing more than a comparison of the stoichiometric properties of the two substances. Schmitz was unable to free his inhibitor from considerable amounts of ammonium sulphate, and his estimate of enzyme inhibition was based on the increase in non protein nitrogen following relatively short hydrolysis of casein by trypsin. If the substance obtained by this method from human plasma is the specific inactivator for plasma trypsin, it would be expected to lose activity in the presence of streptokinase, which was not the case. From the method of fractionation employed it might reasonably be concluded that the substance is part of the serum albumin which has escaped precipitation with the blood, but is partly denatured.

SUMMARY

1 Trypsin and trypsin inhibitors can be assayed viscosimetrically with gelatin as a substrate, using the flow-time index relationship developed by Swyer & Emmens (1947) for the assay of hyaluronidase. Some of the principles involved are discussed.

2 The serum trypsin inhibitor is relatively stable and following fractional precipitation or electrophoresis it is associated with all fractions though mainly with the most soluble albumin fraction. It cannot be removed or destroyed by ether extraction, but is easily damaged by chloroform. It is sensitive to increased hydrogen ion concentration and is lost slowly, if at all, on storage.

3 A small amount of inhibitory material was obtained from human and sheep blood using the method described by Schmitz (1938). The human material was not affected by the addition of streptokinase and it is doubtful if it has the role assigned to it by this author.

4 There is a marked species variation between the antitryptic power of different animal sera, but little variation between the sera of individual members of the same species.

5 No increase was found in the antitrypsin level of rabbit sera following feeding of trypsin and a slight increase after repeated daily injections of trypsin. No change was observed in the inhibitory power of guinea pig sera following protein sensitization either before or after the production of anaphylaxis.

6 The sera of certain persons suffering from tuberculosis showed a marked increase in inhibitory

power as did the sera of all women examined in the terminal stages of pregnancy.

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Protease Inhibitors

2 BACTERIAL PROTEASES AND THEIR INHIBITORS

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Inhibition of bacterial proteases by normal or immune sera has been studied by various authors, although the results are not always in agreement, probably because of the failure to recognize that the filtrates might contain several proteases, only one of which was inhibited by normal serum. In addition, sensitivity to inhibitor might vary according to the organism and type of culture used. von Dungern (1898) found that the hydrolysis of gelatin by filtrates of *Bacillus anthracis*, *Vibrio cholerae* and *Staphylococcus aureus* was inhibited by immune but not by normal serum. Bertiau (1914) found that normal human serum had no inhibitory action on the proteases of *Bacillus subtilis* and *Pseudomonas pyocyanea*, although specific immune serum inhibited. Blanc & Pozerski (1920) showed that gelatin hydrolysis by filtrates of *Clostridium sporogenes* was inhibited by normal horse serum,

which had no action on filtrates of *Cl histolyticum*. Pozerski & Guelin (1938) showed that raw egg white had similar properties with regard to these filtrates. A more comprehensive study by Maschmann (1937*a, b*, 1938*a, b, c, d, e*) showed that at least three extracellular proteases could be recognized, and that a bacterial filtrate might contain one or more of these depending on the organism and method of preparation. A summary of Maschmann's findings is given in Table 1.

Smith & Lindsley (1939), in a study of the hydrolysis of gelatin by filtrates of various Clostridia, showed that, whilst normal serum inhibits proteases of non-pathogenic bacteria, it has no action on those of pathogens such as *Cl histolyticum*, *Cl welchii* and *Cl oedematiens*. Filtrates of a rough strain of *Cl histolyticum* were, however, inhibited by rabbit serum, but experiments showed that,

Table 1 *Classification of bacterial proteases according to Maschmann (1937a, b, 1938a, b, c, d, e)*

Protease	Organism	Substrate	Activator	Inhibition by normal serum
(1) 'Pyocyanus protease'	<i>Ps pyocyanea</i> * <i>Chromobact prodigiosum</i> * <i>Ps fluorescens liquefaciens</i> * <i>B mesentericus</i> *	All proteins and peptones	Not required	+
(2) 'Gelatinase'	<i>Cl welchii</i> <i>Cl septicum</i> <i>Cl histolyticum</i> <i>Cl chauvei</i>	Gelatin only	Not required	-
(3) 'Anaerobiase'	<i>Cl welchii</i> <i>Cl septicum</i> <i>Cl histolyticum</i> <i>Cl botulinum</i>	All proteins	Requires low redox potential (SH compounds best)	-

* These organisms are aerobes all others listed above are anaerobes

whilst the inhibitory power of serum against the filtrates of a rough strain of *Cl histolyticum* was lost on storage or on treatment with ammonium sulphate to half saturation, there was no corresponding loss when tested against trypsin. Electrophoretic separation of normal rabbit serum showed that the inhibitory power against trypsin lay entirely in the albumin fraction, whilst that against the protease produced by a rough strain of *Cl histolyticum* was present also to some extent in the β -globulin fraction.

Some explanation of these findings can be found in the studies of Maschmann (1937a, b, 1938a, b, c, d, e), who showed that the pathogenic Clostridia, *Cl welchii*, *Cl histolyticum*, produce a specific gelatinase not inhibited by serum. Bidwell & van Heyningen (1948) have since confirmed the substrate specificity of the *Cl welchii* gelatinase.

EXPERIMENTAL

Culture filtrates The cultures of *Staph aureus*, *Chromobacterium prodigiosum* and *Ps pyocyanea* were grown 18–24 hr in digest broth continuously shaken. Those of *B anthracis* and *V cholerae* were grown in Roux bottles on agar for 18–24 hr and washed off in saline. *Cl bifementans* was grown on the medium of Macfarlane & Knight (1941). *Cl welchii* Types A and D, and *Cl histolyticum* were grown 8–18 hr in a meat infusion broth containing 0.6% (w/v) glucose and 0.001M thioglycolic acid. The *Cl welchii* Type B culture was grown in a glucose peptone broth containing thioglycolic acid and sodium glycerophosphate. All cultures with the exception of those grown on agar were filtered free of organisms, those grown in liquid media were concentrated 5–30 times by pressure dialysis, and all were finally dialyzed against saline and stored under toluene at 4° or frozen.

Estimation of enzyme and inhibitor activity The majority of estimations were made by the viscosimetric technique already described (Duthie & Lorenz, 1949). A standard preparation of crystalline trypsin which was assigned the arbitrary value of 20,000 units/mg was the standard of

comparison. The casein hydrolysis experiments described in Table 2 had the enzyme concentration given in the second column of the table in a total of 1 ml of 1% casein, and were incubated 3 hr at 37°, after which the mixtures were precipitated with 2 vol of 5% (w/v) trichloroacetic acid. The increase in acid soluble 'tyrosine' against control mixtures not incubated was estimated by the Folin and Ciocalteu phenol reagent.

All inhibitors were incubated for 10 min at 37° with the enzyme before the addition of substrate. Milk clotting times were estimated by adding equal volumes of fresh milk to the enzyme concentration given. Collagenase activity was measured as described by Oakley, Warrack & van Heyningen (1946).

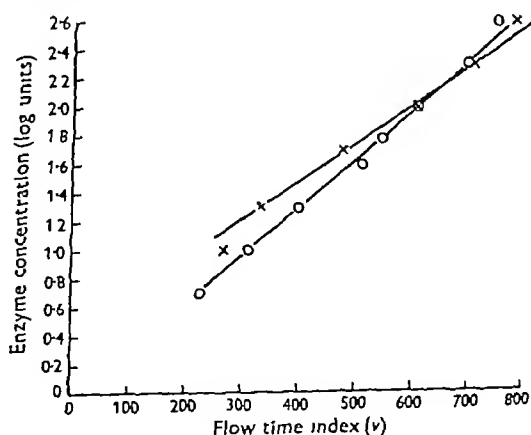


Fig 1 Relationship between flow time index (v) and logarithm of enzyme concentration for two bacterial filtrates: x, *Ps pyocyanea*; o, *Staph aureus* (100 units of bacterial filtrate \equiv 5 μ g of crystalline trypsin, i.e. a flow time index of approximately 600).

RESULTS

Measurement of proteolytic activity

Effect of bacterial proteases on gelatin The examination of a number of bacterial proteases (Fig 1) showed that the flow time index (v) plotted against

the logarithm of enzyme concentration gave a straight line over at least part of the range $v=600-200$. The slope of the line in the case of certain organisms approximates to that for trypsin, lying between $1/380$ and $1/450$ (Duthie & Lorenz, 1949), but in the case of other organisms, except *Cl welchii* Type B, it lay between $1/250$ and $1/300$.

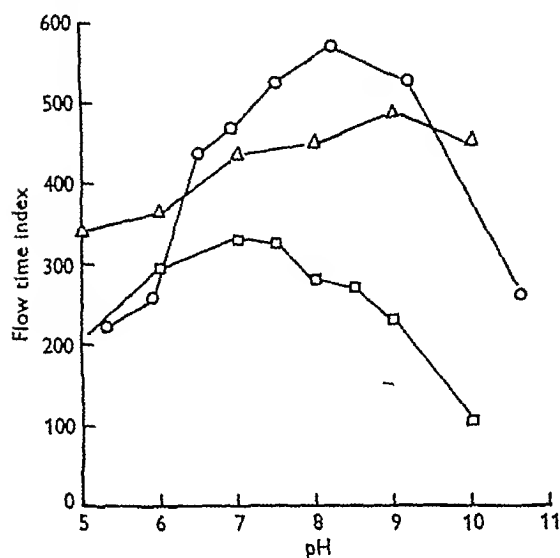


Fig 2 Effect of pH on enzyme activity of filtrates of aerobic bacteria as measured by the flow-time index Δ, *Chromobact prodigiosum*, □, *Staph aureus*, ○, *V cholerae*

(Fig 1, Table 2) Thus dilution of these latter decreased the rate of gelatin hydrolysis much less than in the case of trypsin. Quantitative viscosimetric comparisons of protease activity between these two types of filtrate were, therefore, impossible, and all solutions used in Table 2 were standardized so as to have a v value of about 600 after 20 min hydrolysis of gelatin.

Effect of pH The filtrates chosen for comparison were selected from those bacteria which showed well-marked proteolytic activity in crude unconcentrated filtrates. The majority of such filtrates showed optimal activity between pH 7 and 9 (Figs 2, 3), although a filtrate of *Cl welchii* Type A

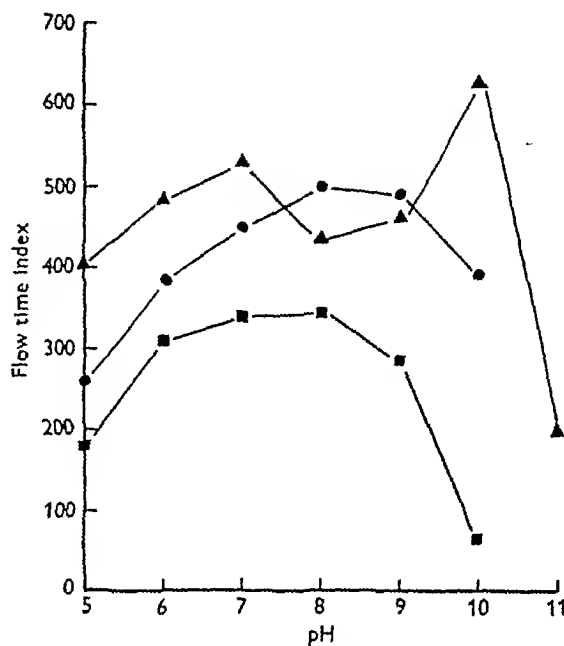


Fig 3 Effect of pH on enzyme activity of filtrates of anaerobic bacteria as measured by the flow-time index Δ, *Cl welchii* Type A filtrate, ■, *Cl welchii* Type B filtrate, ●, *Cl histolyticum*

showed two optima, one at pH 7 and the other at pH 10, indicating the presence of two enzymes. Walbum & Reymann (1934) obtained similar curves for this organism, although their optima were slightly lower. All filtrates showed good activity around pH 7.6, which was that used in the majority of experiments.

Table 2 Effect of the same concentration of different bacterial filtrates on different substrates and a comparison of the inhibitory activity of normal sheep serum

Organism	Gelatinase activity (flow time index)		Slope of gelatin activity curve	Activity against casein measured as μg 'tyrosine' liberated/ml digest		Milk clotting time (min)
	Enzyme alone	Enzyme and 10% (v/v) sheep serum		Enzyme alone	Enzyme and 10% (v/v) sheep serum	
<i>Staph aureus</i> (113344)	612	406	$1/270$	102	48	—
<i>B anthracis</i>	603	0	$1/250$	110	0	18
<i>Chromobact prodigiosum</i>	646	0	$1/440$	118	18	15
<i>Ps pyocyanea</i>	620	0	$1/380$	111	0	30
<i>B megatherium</i>	598	0	$1/260$	100	0	3
<i>Cl welchii</i> Type A	600	550	$1/450$	0	0	—
<i>Cl welchii</i> Type B	570	0	$1/110$	80	39	3
<i>Cl bisfermentans</i>	613	0	$1/280$	84	0	4
<i>Cl histolyticum</i>	645	325	$1/410$	66	0	—
Crystalline trypsin	606	0	$1/400$	81	0	—

Serum inhibition

Action of serum The addition of serum to a final concentration of 10 % (v/v) affected the hydrolysis of both gelatin and casein in the case of all the bacterial filtrates studied (Table 2). Complete inhibition of gelatin hydrolysis was found in the case of four of the five filtrates of aerobic bacteria and in two of the anaerobic bacteria. Wide variation in sensitivity to serum was encountered among those completely inhibited by 10 % sheep serum, a 50 % inhibition was produced in 100 units of a filtrate of *Chromobact prodigiosum* by a final dilution of 1.25 % serum, whereas the same effect was produced in 100 units of a filtrate of *Ps pyocyanea* by as little as 0.05 % serum. The inhibitory power of different animal sera varied according to the species (Table 3), sheep being very much more inhibitory as

Table 3 Comparison of inhibitory effect of normal animal sera on a culture filtrate of *Ps pyocyanea* as measured by flow-time index

Flow time index	Sera			
	Rabbit	Human	Horse	Sheep
	385	390	330	190

(Filtrate 100 units/ml sera 0.16 % (v/v))

in the case of crystalline trypsin (Duthie & Lorenz, 1949). The interaction between serum and a filtrate of *Ps pyocyanea* was immediate, there being no increase in inhibition if the mixture was incubated for varying periods prior to the addition of gelatin.

Effect of age and heating on serum Specimens of horse and human sera kept at 4° rapidly lost part of their inhibitory power against bacterial filtrates, though this was not so noticeable in the case of sheep serum. Table 4 shows that, in a comparison

Table 4 Comparison of loss in inhibitory effect of stored normal sera on a bacterial filtrate (*Ps pyocyanea*) and on crystalline trypsin

(Sera stored 1 year at 4° Enzyme 100 units/ml.)

	Loss in inhibitory power (%)	
	Human serum	Sheep serum
Filtrate of <i>Ps pyocyanea</i>	66	80
Crystalline trypsin	30	Nil

of fresh and stored human and sheep sera, the loss in inhibitory activity is very much greater when measured against a bacterial filtrate than against crystalline trypsin. The comparison is based on the assumption that the stored sera had originally the

same inhibitory power as the fresh sera used. Even if this were not the case, the figures show clearly that inhibitory activity against trypsin and a bacterial filtrate are unrelated, since the fresh sheep serum was equal in inhibitory power to the old serum when measured against crystalline trypsin, though five times as active when measured against the bacterial filtrate. The inhibitor against bacterial filtrates was very sensitive to age and to acidity, more than 90 % of the activity of sheep serum being lost after 2 hr at 37° and pH 4.6 or 30 min at 70° in neutral solution. The loss in activity against trypsin under these conditions was less than one third (Duthie & Lorenz, 1949).

Salt fractionation Precipitation with ammonium sulphate provided further proof (Table 5) that the serum inhibitor against a bacterial filtrate was different from that against crystalline trypsin since

Table 5 Comparison of inhibitory power of sheep serum fractions measured against 100 units of crystalline trypsin and a culture filtrate of *Ps pyocyanea*

(Fractions separated by $(\text{NH}_4)_2\text{SO}_4$ and activity expressed as percentage of inhibitory activity of whole serum)

	Globulin	Albumin precipitated by complete saturation with $(\text{NH}_4)_2\text{SO}_4$ at pH 4.2 (Fraction C_1)
Crystalline trypsin	20	45
<i>Ps pyocyanea</i>	38	28

the more soluble albumin fraction C_1 , precipitated by full saturation with ammonium sulphate at the isoelectric point, contained nearly half the original activity against trypsin but only 3 % of the original activity when measured against the bacterial filtrate. Similar results were obtained with fractions separated electrophoretically. Thus while both globulin and albumin fractions are inhibitory, though in different degree, to crystalline trypsin, the bulk of the inhibitory action against bacterial proteases is associated with the globulin.

Effect of other inhibitors

Egg white and soya bean Neither soya bean trypsin inhibitor nor pancreatic trypsin inhibitor had any action on the proteases of either *Staph aureus*, *Ps pyocyanea* or *Chromobact prodigiosum*. Raw egg white resembled serum with regard to the varying sensitivity of different bacterial filtrates (Table 6). Ovomucoid purified according to the method of Lineweaver & Murray (1947) was active against 100 units of crystalline trypsin at 1, 2,000,000, but was without activity against bacterial filtrates at 1, 1000.

Table 6 Comparison of sensitivity of different bacterial filtrates to serum and to egg white

	Concentration of inhibitor (%)	Inhibition of proteolysis (%)		
		<i>Ps pyocyanea</i>	<i>Chromobact prodigiosum</i>	<i>Cl welchii</i> Type A
Human serum	1.25	100	50	Nil
Egg white	2.5	62	33	Nil
Soya bean	0.1	Nil	Nil	Nil

Specific antisera When specific antiserum is added to a constant amount of a culture filtrate of *Staph aureus* or of *Cl welchii* Type A, and the flow-time index is plotted against the units of antiserum added, the curves obtained are not dissimilar from those obtained by the addition of normal serum to either crystalline trypsin (Duthie & Lorenz, 1949) or to filtrates of bacteria (Fig 4). The viscosimetric

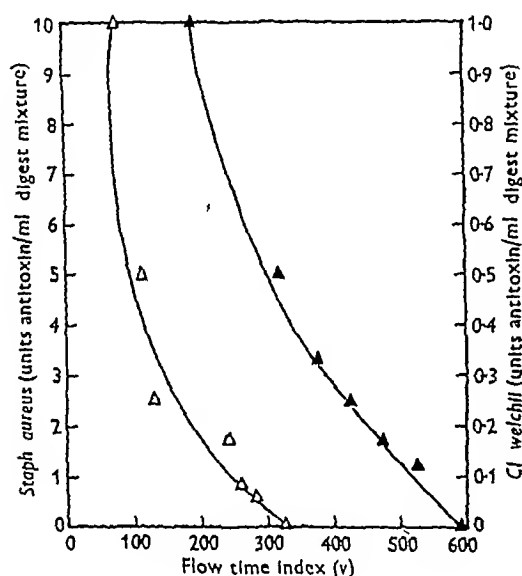


Fig 4 Effect of increasing amounts of specific antiserum on filtrates of *Staph aureus* and *Cl welchii* Type A. (Staphylococcal antiserum was a globulin fraction, *Cl welchii* antiserum was a pepsin digested globulin) Δ , *Staph aureus*, \blacktriangle , *Cl welchii*

method can thus be used in the estimation of the antiprotease component of any antiserum. Both staphylococcal and *Cl welchii* Type A antisera were specific in their antiprotease activity and the *Cl welchii* Type A antiserum did not neutralize the proteases in *Cl welchii* Type B filtrates.

Comparison of filtrate activities

Table 2 shows that for all five filtrates of aerobic bacteria examined there was a very good correlation between casein and gelatin hydrolysis, comparing the v value and acid soluble 'tyrosine' liberated, indicating that the same enzyme is probably involved. The filtrates from *Clostridia* showed a

much greater degree of variation and, in addition, a lower ratio of casein to gelatin hydrolysis. Of these four filtrates, only *Cl welchii* Type A failed to hydrolyze casein, and to hydrolyze gelatin in the presence of serum. The hydrolysis of gelatin by a filtrate of *Cl histolyticum* was only partly inhibited by serum, and both filtrates presumably contain a specific gelatinase. These two filtrates attacked native collagen in the form of muscle and tendon, but collagenase and gelatinase properties are not due to the same enzyme, since two purified preparations of collagenase received from Miss E. Bidwell had lost completely their power of attacking native collagen following 4 months' storage at 4°, while retaining their gelatinase properties unimpaired.

None of the filtrates in Table 2 showed increased proteolysis in the presence of thioglycolic acid. Only certain filtrates clotted milk, and then the rate of reaction was not related to protease activity as measured either on gelatin or casein. The clotting of milk was inhibited by normal sera and especially by the globulin fraction. The albumin fraction used in the experiment of Table 5 had little or no inhibitory effect.

DISCUSSION

In contrast to serum antitrypsin, which is associated with the more soluble albumin fraction, the serum inhibitor of bacterial proteases is associated mainly with the globulins. This property is also much more labile than in the case of serum antitrypsin, but it does not disappear completely on storage as stated by Smith & Landsley (1939). Likewise, whilst considerable differences exist between the inhibitory power of the sera of different species, it is never absent as claimed by Maschmann (1937b), and such discrepancies as have been reported are due either to varying sensitivity of the proteases tested or to failure to use fresh serum, since the inhibitory titre of horse serum can fall very rapidly even at 4°. The rapid combination of the serum inhibitor of bacterial proteases with enzyme resembles that of an antigen-antibody reaction, and further distinguishes this inhibitor from serum antitrypsin. Todd (1947) has found that the serum of about 5% of normal horses and 25% of normal humans investigated contained

an inhibitor for a streptococcal protease which he studied. The inhibitor also lay in the globulin fraction and suggested some form of 'natural antibody'. Wide variations in antiprotease titre, such as were found by Todd, were not noticed in the present studies, but sufficient work has not been done on this point. All human and animal sera investigated inhibited susceptible bacterial proteases, and the inhibition would, therefore, appear to be part of a non-specific immunity reaction.

Recently Haas (1946) has studied the inhibitory action of serum on hyaluronidase both of testicular and of bacterial origin. Haas finds that, like the reaction between serum antitrypsin and trypsin, the amount of inhibition increases with time of contact of enzyme and serum prior to incubation with substrate. Similarly, the inhibitor is destroyed by short treatment with acid and by heating to 53° for 15 min. It is apparently assumed that the same substance is responsible for the inhibition of both testicular and bacterial hyaluronidase, since the source of the enzyme used in these experiments is not specified. On the basis of these findings, Haas concludes that the serum inhibitor of hyaluronidase is an enzyme which he terms antivasin 1. Since the inhibitory properties of serum for both trypsin and bacterial proteases are analogous to and closely resemble those antihyaluronidase properties described by Haas, one might equally regard them as being due to an enzyme. While such a possibility might be considered, it is felt that the evidence produced by Haas is insufficient.

The filtrates of *Cl. welchii* Type A and *Cl. histolyticum* clearly contain the substrate-specific gelatinase described by Maschmann (1937*a, b*, 1938*b*), which is only partly inhibited by very large

amounts of normal serum such as 10% (v/v) used in Table 2. Although gelatinase is closely associated with collagenase in the purification process used by Bidwell & van Heyningen (1948), the two are not identical, since collagenase activity alone is lost on storage or on dialysis at pH 10.0 (Bidwell personal communication).

SUMMARY

1 The flow-time index relationship described by Swyer & Emmens (1947) can be used for the viscosimetric assay of bacterial proteases and their inhibitors.

2 The serum inhibitor active against bacterial proteases is a labile component of the globulin fraction, in contrast to the relatively more stable serum antitrypsin, which is found mainly in the albumin fraction. The serum of different animal species varies in its inhibitory power.

3 Bacterial proteases vary in their sensitivity to the serum inhibitor; the specific gelatinase found in certain *Clostridia* being relatively insensitive. Filtrates sensitive to serum are also inhibited by egg white, but all are unaffected by ovomucoid soya bean or by pancreatic trypsin inhibitors.

4 *Cl. welchii* Type A gelatinase is inhibited by specific antiserum and the potency of different antisera can be compared viscosimetrically. The gelatinase activity is independent of collagenase activity.

The authors wish to thank Mrs A. A. Miles for the *Cl. bifementans* filtrate, Dr G. P. Gladstone for the filtrate of *B. anthracis*, Miss E. Bidwell for the purified specimen of collagenase, Mr M. V. Stack for protein estimations and Dr R. Kekwick for electrophoretic separation of serum fractions.

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The Measurement of Radioactive Sulphur (^{35}S) in Biological Material

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The only known radioactive isotope of sulphur which is suitable for biochemical investigations is ^{35}S . During the period 1941–5, ^{35}S was used in this laboratory as a tracer isotope in biochemical studies of mustard gas (di-2-chloroethyl sulphide), certain mustard gas derivatives, and 2,3-dimercaptopropanol (BAL). These investigations were made possible as a result of ^{35}S being made available for the work from sources in the U.S.A., and through the kindness of Dr F. C. Henriques, jun. of Harvard University, who supplied the radioactive material in the form of mustard gas. It was during the course of the work with radioactive mustard gas that the present method of measuring ^{35}S in biological material was developed.

^{35}S has a half life period of 87.1 days (Hendricks, Bryner, Thomas & Ivie, 1943) and it emits β particles with a maximum energy of 0.120 MeV (Kamen, 1941). The low energy β particles are easily stopped by relatively thin layers of absorbing material, and in order to measure ^{35}S accurately it is necessary to make corrections for the absorption of the radiation by the radioactive material itself. Correction for self-absorption of the radiation is facilitated by the separation of the isotope as a constituent of a sulphur compound. The radioactivity of this compound is then determined in an apparatus sufficiently sensitive to measure soft radiation. Such a procedure can be developed by modifying a gravimetric method for the determination of sulphur in such a way that the final precipitate is collected in a form suitable for the measurement of its radioactivity. A relation can then be obtained experimentally between the thickness of the precipitate in this form and its radioactivity.

Several methods have been described in which the material containing ^{35}S is oxidized and the sulphate is precipitated as the barium or benzidine salt, the radioactivity of which is measured. Among the oxidation procedures which have been used are the Pirie (1932) method (Tarver & Schmidt, 1939; Bournsnel, Francis & Wormall, 1946), the Carus method (Henriques, Kistakowsky, Margnetti & Schneider, 1946), oxidation with concentrated nitric acid and hydrogen peroxide (Seligman & Fine, 1943), and oxidation with sodium carbonate and sodium peroxide (Dziewiatkowski, 1945).

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The first method used in this laboratory (Young, 1941) for the separation of sulphur in biological material for radioactivity measurements was based on the Masters (1939) modification of the ter Meulen (1922) procedure for determining sulphur. The type of apparatus required and the amount of attention needed for the conduct of this method rendered it unsuitable for the performance of the large number of analyses which had to be carried out and it was eventually abandoned. Various modifications of the nitric acid-perchloric acid oxidation procedure (Pirie, 1932; Masters, 1939) were also tested, but in our hands they yielded low results when used for the determination of the sulphur content of some of the compounds being studied (e.g. mustard gas, thiodiglycol).

The procedure finally adopted (Patrick & Young, 1943; McCarter & Young, 1944) was based on that originally described for the analysis of organic compounds by Carus (1860). A study was made of the conditions which must be observed if this method is to be applied to biological material. After the material had been oxidized, the sulphate was precipitated as benzidine sulphate by a procedure based on that described by Niederl, Baum, McCoy & Kuck (1940) for the microdetermination of sulphur in organic compounds. The benzidine sulphate precipitate was collected by a method designed to reduce, as far as possible, self absorption of the radiation by the compound during the measurement of its radioactivity. In order to correct for the small amount of self-absorption which did occur, the deposit of benzidine sulphate was weighed, and all radioactivity measurements were referred to the same weight of benzidine sulphate. The decision to separate the sulphate as the benzidine salt rather than as barium sulphate was based mainly on the finding that benzidine sulphate yields a uniform deposit suitable for radioactivity measurements much more readily than does barium sulphate.

The radiation from ^{35}S can be measured in several different ways. Henriques *et al.* (1946) compared the Lauritsen quartz fibre electroscope and a counter with a Geiger tube with a very thin window, and found them to be equally satisfactory on the basis of accuracy and sensitivity, although these workers indicated a preference for the electroscope for the routine measurement of ^{35}S . The apparatus used in the present work was an ionization chamber with

a direct current amplification system employing an FP-54 tetrode valve. This valve has been described by Metcalf & Thompson (1930). When it is operated in conjunction with a galvanometer, currents of the order of 10^{-14} amp can be measured with comparative ease, and currents as small as 5×10^{-18} amp can be detected (DuBridge, 1931).

EXPERIMENTAL

Conversion of the sulphur of organic compounds and biological material to benzidine sulphate

Experiment showed that 1–2 mg S yielded an amount of benzidine sulphate (8.8–17.6 mg) suitable for radioactivity measurements, and it was necessary to oxidize an amount of material which contained this weight of S if the addition of non-radioactive sulphate to the sample was to be avoided. In order to do this it was necessary to know the approximate S content of the material being analyzed. There is little information on the total S content of tissues to be found in the literature. Kambayashi (1929) reported mean values of 2.22 and 2.42 mg, respectively, for the S content/g of fresh liver and muscle of rats (body wt, 180–250 g), and similar values for the tissues of other animal species have been reported (Silberstein, 1934; Brown & Klauder, 1935; Masters & McCance, 1939; Sherman, 1941). It appeared probable, therefore, that it would be necessary to develop an oxidation procedure which could be applied to 0.3–0.5 g of fresh tissue. This was confirmed by experience.

Experiments were conducted in which the size of the Carius bomb, the amount of HNO_3 , and the time and temperature of heating the bomb were varied until a satisfactory set of conditions was found for the oxidation of 0.3–0.5 g of tissue. The methods used for the evaporation of the contents of the bomb and for the precipitation of the sulphate with benzidine hydrochloride were based on those used by Niederl *et al.* (1940) for the microdetermination of S in organic compounds. Benzidine sulphate separates well when centrifuged, and when ^{35}S was to be measured, the benzidine sulphate was separated and washed in a centrifuge tube before depositing it on a Petri dish for radioactivity measurements. When the method was applied to the gravimetric determination of S, the benzidine sulphate was collected on a microfilter with a fritted disk, washed, dried and weighed as described by Niederl *et al.* (1940). A detailed account of the procedure is given below.

Procedure. The following reagents were used: Red fuming HNO_3 (Baker's C.P. Analyzed Sp gr 1.59–1.60).

NaBr Analytical reagent grade

Benzidine hydrochloride reagent. To 5 g of pure benzidine hydrochloride were added 40 ml of N-HCl and enough 50% (v/v) aqueous ethanol to give 250 ml. The solution was heated until it was just boiling. It was then cooled, filtered and stored in a dark glass stoppered bottle. The solution was filtered from time to time.

Ethanol, 50 and 95% (v/v). These reagents were prepared by diluting pure ethanol with water, and were tested to ensure that they did not yield a significant amount of residue when evaporated to dryness.

Great care was taken in the cleaning of all glassware used in the measurement of ^{35}S . This was especially important in the case of glassware which had been used in the analysis of strongly radioactive material.

The general procedure adopted for preparing and sealing the Carius bombs was that described by Niederl *et al.* (1940). The bombs were made from Pyrex glass tubing with an internal diameter of 2.2 cm and an external diameter of 2.5 cm. The length of a bomb was based on the quantity and nature of the material to be oxidized, and in general the length was between 30 and 50 cm. Whenever possible, the amount of material oxidized was such that its S content was 1–2 mg. When it appeared probable that a sample of material which was being analyzed for its ^{35}S content contained less than 1 mg S, sufficient non-radioactive Na_2SO_4 was added to raise the S content of the benzidine sulphate precipitate to at least 1 mg. The sample was introduced into the bomb together with 5–10 mg of NaBr and 1.2 ml of red fuming HNO_3 (in some analyses it was necessary to use larger amounts of HNO_3 , and under these conditions bombs of greater length than 50 cm were used). The open end of the bomb was drawn out to a thick-walled capillary and sealed immediately after the introduction of the reagents. The bomb was then heated at 300° in a Carius furnace for not less than 4 hr. After the bomb had been allowed to cool in the furnace, the tapered portion was heated gently to drive into the main part of the bomb any liquid which had condensed in the capillary. In order to release the pressure inside the bomb the tip of the capillary was then heated with a small hot flame until an opening was forced by the compressed gases. A scratch mark was then made around the bomb just below the tapered portion, and the bomb was cracked around this mark by applying a hot glass rod to it. The tapered portion was separated from the main part of the bomb and washed with water. These washings, together with the contents of the bomb, were washed with water (total vol 25 ml) into an evaporation tube and evaporated to dryness.

The evaporation tube consisted of a Pyrex boiling tube (20×2.5 cm) with a short glass side arm attached at right angles 4 cm from the mouth. The evaporation tube was closed by a cork (a new cork was used for each analysis) carrying an inverted L-shaped inlet tube with a constriction at the lower end. A short length of rubber tubing with a screw clip was attached to the upper end of the inlet tube. The evaporation tube was clamped in a boiling water bath, the side arm connected through a trap to a water pump, and a current of air drawn through the tube. The flow of air was regulated by means of the screw clip on the inlet tube, and the position of the inlet tube was adjusted so that its lower end was just above the surface of the solution. After the solution had evaporated to dryness the glass inlet tube was washed with water (total vol 3 ml). These washings were collected in the evaporation tube and served to dissolve the residue. Two ml of 95% (v/v) ethanol were then added with shaking, followed by 1 or 2 ml of benzidine hydrochloride reagent (the larger amount of this reagent was used when ever it appeared probable that the sample contained more than 2 mg S). The precipitate of benzidine sulphate was stirred and allowed to stand for 30 min. The contents of the evaporation tube were then transferred to a tapered 15 ml centrifuge tube. The evaporation tube was washed with small portions of 95% ethanol (total vol 3 ml) and these were added to the contents of the centrifuge tube. The precipitate after centrifuging was suspended in 10 ml of 95% ethanol and recentrifuged. The precipitate was then again suspended in 95% ethanol, and transferred to a weighed Petri dish, 9 cm in diameter. The precipitate was evaporated

ated to dryness by holding the Petri dish over an electric heater. During the evaporation the dish was manipulated in such a way that an even deposit of benzidine sulphate was obtained, covering the bottom of the dish. After cooling the dish was placed overnight in a desiccator. It was weighed and was then covered with its lid until the radioactivity of the deposit was measured.

The following procedure was used when the ^{35}S content of the analyzed sample was likely to exceed that which could be measured accurately with the ionization chamber apparatus. The aqueous solution obtained after dissolving the residue in the evaporation tube was made to a known volume. A measured portion of this solution was then diluted to 3 ml, non-radioactive Na_2SO_4 was added when necessary, and the addition of 95% ethanol and precipitation of the benzidine sulphate were carried out as described above.

Further details of the application of the method to the measurement of ^{35}S in organic compounds, tissues and excreta are given below.

Analysis of organic compounds When the method was applied to a compound containing ^{35}S , the sample (liquid or solid) was weighed in a small stoppered glass tube on a microbalance. The NaBr and red fuming HNO_3 were placed in the bomb, the stopper was removed from the tube containing the sample, the tube was introduced carefully into the bomb, and the bomb was sealed without delay.

Analysis of tissues Rat tissues were placed in stoppered weighing bottles immediately after removal from the animal. A representative sample (0.3–0.5 g) of each tissue was weighed, transferred to a bomb containing NaBr, red fuming HNO_3 was added, and the bomb was sealed.

Two procedures were used for the measurement of ^{35}S in blood. When the concentration of ^{35}S in the blood was such that accurate results could be obtained by the analysis of 0.2 ml samples of blood, this amount was introduced into a bomb together with 1 mg S (as non-radioactive Na_2SO_4), and the analysis completed as described above. When it was necessary to analyze larger samples, 1 ml of blood, 1 ml of water and 1 ml of red fuming HNO_3 were placed in a bomb. The bomb was left unsealed and its contents were evaporated to small volume on an air bath. NaBr and 1 ml of red fuming HNO_3 were then added, the bomb was sealed, and the analysis then completed in the usual way.

When the whole carcass of a rat was analyzed, it was placed in a beaker (2 l) and red fuming HNO_3 (approx. 0.67 ml/g of carcass) was added. The mixture was stirred from time to time and all of the carcass, with the exception of some fatty material, dissolved in the HNO_3 in a few hours at room temperature. The mixture was then transferred to a separating funnel and the HNO_3 solution was separated from the fatty material. The volume of the HNO_3 solution was measured, 0.5 ml. was transferred to a bomb which contained NaBr and 1 ml of red fuming HNO_3 , and the analysis completed as described. The fatty material was stirred vigorously with red fuming HNO_3 (about 50 ml of acid were used for the fatty material from a rat weighing 150 g), the volume of the suspension was measured, and a 1 ml sample was sealed in a bomb which contained NaBr and 0.5 ml of red fuming HNO_3 .

Analysis of excreta In order to determine the total ^{35}S content of urine, the volume of the urine was measured and 0.2 ml was sealed in a bomb with 1 mg S (as non-radioactive Na_2SO_4), NaBr and 1 ml of red fuming HNO_3 . For

the analysis of faeces, the material was first ground in a mortar, and a weighed sample was then oxidized in the usual way.

Gravimetric determination of sulphur The procedure differed from that already described in the following respects. The solution obtained by dissolving the residue in the evaporation tube was filtered to remove dust particles and any other insoluble material. The benzidine sulphate was precipitated under the conditions already described. It was then filtered on a microfilter with a fritted disk, washed with small portions of 50% (v/v) ethanol (total vol. 10 ml), and dried to constant weight at 100–102°.

Measurement of the radioactivity of benzidine sulphate samples

Apparatus The apparatus consisted of an ionization chamber and an FP-54 electrometer circuit*. The circuit used was based on that described by DuBridge & Brown (1933). The FP 54 valve (General Electric Co., Schenectady, New York) was housed in an inverted position in an evacuated brass container directly above the ionization chamber. The ionization chamber was made of brass and was cylindrical in shape (internal dimensions: diameter, 11 cm, height, 7 cm). It consisted of an upper and a lower part which could be separated to permit the introduction of the Petri dish carrying the radioactive sample. The control grid of the valve was connected to the central electrode of the ionization chamber and a resistor of $10^{12} \Omega$, situated inside the housing of the valve, was connected between the control grid and earth. The sensitivity of the electrometer circuit was controlled by using an Ayrton shunt in conjunction with the galvanometer (no. 2500 g, Leeds and Northrup, Philadelphia, Pa.).

Current flow in the circuit was adjusted so that the galvanometer showed no deflection when no radioactive material was present in the ionization chamber. When a radioactive sample was introduced into the chamber a galvanometer deflection was produced which reached its equilibrium position in 10–15 min. The minimum amount of radioactivity measurable with the apparatus was considered to be that which gave rise to a deflection of 50 mm when the galvanometer was operated at its maximum sensitivity. A β ray standard (prepared from uranium oxide) with an activity of 0.01 μC , produced a deflection of 250 mm.

^{35}S standards The ^{35}S used in the present work was received in the form of pure mustard gas (m.p. 14.2–14.4°). The ^{35}S content of 5 mg of this mustard gas was considered arbitrarily to be 1000 units. The number of atoms of ^{35}S in this material was a very small fraction of the total number of S atoms, and as the S content of non-radioactive mustard gas is 20.2%, 1 unit corresponded closely to the $^{35}\text{S}/\mu\text{g}$ of mustard gas S.

In order to compensate for variations in the sensitivity of the apparatus used for radioactivity measurements, and to avoid making corrections for decay, standard ^{35}S samples were measured on each half day that the radioactivity of unknown samples was measured. Each standard consisted

* The use of this apparatus was suggested to us by Dr N. B. Keevil, Department of Physics, University of Toronto, and with his guidance the apparatus was assembled by one of us (M. E.).

of 1 mg S in the form of benzidine sulphate, and it contained a known number of units of ^{35}S , i.e. it contained the ^{35}S from a known weight of the parent specimen of radioactive mustard gas

Relationship between galvanometer deflexion and the ^{35}S content of the benzidine sulphate sample The ionization chamber apparatus was adjusted so that, as far as possible, a linear relationship existed between the galvanometer deflexions and the activities of the samples. The galvanometer deflexions produced under these conditions by a series of ^{35}S standards are shown in Table 1

Table 1 *Galvanometer deflexions produced by a series of ^{35}S standards*

^{35}S in sample (units)	Galvanometer shunt	Galvanometer deflexion (mm)
0.005	1	84
0.010	1	167
0.020	1	330
0.050	0.1	72
0.100	0.1	141
0.200	0.1	296
0.50	0.01	91
1.00	0.01	190
2.00	0.01	433

When the data in Table 1 are plotted it is seen that an almost straight-line relationship existed between the amount of ^{35}S and the galvanometer deflexions for those measurements in which the 1 or 0.1 shunt was used. There was divergence from such a relationship, however, for the data obtained with the samples which required the use of the 0.01 shunt.

Self absorption of β particles by benzidine sulphate samples The low energy of the radiation emitted from ^{35}S results in absorption of an appreciable fraction of the β particles by the benzidine sulphate. In order to determine the influence of the thickness of the sample on a radioactivity measurement, samples of benzidine sulphate were prepared which contained the same amount of ^{35}S , but which differed in

weight. The experiments were carried out in quadruplicate and all the samples were deposited on Petri dishes 9 cm in diameter. The results obtained when the radioactivity of these samples was measured are shown in Table 2.

Table 2 *Self-absorption of β particles by benzidine sulphate samples*

S in sample (mg)	Benzidine sulphate (mg)	Average 'thickness' of sample (mg/sq cm)	Observed radioactivity*
1	8.8	0.14	1.00
2	17.6	0.28	0.95
3	26.4	0.41	0.91
4	35.2	0.55	0.86
5	44.0	0.69	0.81

* Expressed as a fraction of the radioactivity of the samples which contained 1 mg S.

All standard ^{35}S samples contained 1 mg S in the form of benzidine sulphate (8.8 mg), and in order to take self absorption effects into consideration, radioactivity measurements were always referred to this amount of S. Since benzidine sulphate samples of unknown radioactivity always contained at least 1 mg S and were weighed, it was possible by the use of a graph of the data in Table 2 to correct for self absorption effects in the measurement of ^{35}S . This correction was usually small, for the S content of the unknown samples rarely exceeded 2 mg.

Application of the method

Gravimetric determination of sulphur The Carius oxidation-benzidine sulphate method was applied to the gravimetric determination of sulphur in a series of organic compounds including mustard gas, its sulphoxide and sulphone, and thiodiglycol. The results obtained in the gravimetric analyses are shown in Table 3.

Table 3 *Gravimetric determination of sulphur in organic compounds by the Carius oxidation-benzidine sulphate method*

Compound	Wt of compound analyzed (mg)	Sulphur		
		Calc (%)	Found (%)	Recovery (%)
L Cystine	6.36	26.7	27.2	101.9
	5.92	—	27.2	101.9
Phenylmercapturic acid	13.57	13.4	13.7	102.2
	3.19	—	13.6	101.5
<i>p</i> Fluorophenyl L cysteine	6.90	14.9	15.2	102.0
	8.64	—	15.2	102.0
<i>p</i> Fluorophenylmercapturic acid	8.78	12.5	12.6	100.8
	8.94	—	12.7	101.6
Di 2 chloroethyl sulphide	5.17	20.2	20.6	102.0
	5.20	—	20.5	101.6
Di 2 chloroethyl sulphoxide	7.08	18.3	19.0	103.8
	7.46	—	18.7	102.2
Di 2 chloroethyl sulphone	6.66	16.8	17.5	104.2
	9.16	—	17.2	102.3
Di 2 hydroxyethyl sulphide	10.29	26.2	26.5	101.1
	12.98	—	26.6	101.5

The results obtained show that in every analysis the weight of the benzidine precipitate was slightly higher than the theoretical amount. This was not due to the presence of sulphur in the reagents for tests showed that, in the quantities used, the reagents did not contain measurable amounts of sulphur. It seems probable that the error was due to occlusion, for McKittrick & Schmidt (1945) have found that this type of error is liable to occur in the gravimetric determination of sulphate as its benzidine salt. Provided there is complete precipitation of the radioactive sulphate when ^{35}S is being measured (see below), the occlusion of small amounts of material by the benzidine sulphate is, however, of no significance when no other source of radioactivity is present.

Although the method was not developed for the determination of the total sulphur content of tissues and other biological material, the gravimetric procedure was applied to rat tissues in order to find the amounts of benzidine precipitate which these tissues might be expected to yield in the course of the measurement of their ^{35}S content. Four male white rats (body wt 160–185 g) were used for the experiments, and the results expressed as mg of sulphur (mean \pm s.e.) /g of fresh tissue were as follows: liver, 2.55 ± 0.16 , kidney, 2.47 ± 0.11 , heart, 2.89 ± 0.17 , lung, 2.10 ± 0.09 , spleen, 2.20 ± 0.18 , brain, 1.69 ± 0.09 , eye, 2.83 ± 0.12 , stomach, 2.12 ± 0.10 , small intestine, 2.48 ± 0.09 , testis, 1.40 ± 0.05 , leg muscle, 2.47 ± 0.09 , skin (clipped), 2.25 ± 0.09 , carcass (all of the animal that remained after removal of the above tissues), 2.29 ± 0.04 .

For accurate results in the determination of sulphate as the benzidine salt, it is necessary to remove phosphate as a preliminary step (Fiske, 1921, Owen, 1936, McKittrick & Schmidt, 1945). The interfering action of phosphate under the conditions used in the present work was shown by experiments in which 1 mg of sulphur (as Na_2SO_4) was precipitated in the presence of 1, 2, 3, 4 or 5 mg of phosphorus (as KH_2PO_4). The weights of the benzidine precipitates corresponded to 1.05, 1.07, 1.11, 1.32 and 1.86 mg of sulphur, respectively. These results suggest that the gravimetric determination of total sulphur in biological material by this method may give results which are more than 10% in error when the amount of phosphorus present in the material analyzed is three or more times that of the sulphur present. It is noteworthy, however, that the total phosphorus content of a series of rat tissues (liver, kidney, spleen, small intestine, testis and leg muscle) has been found to range from 2.1 to 2.9 mg/g of fresh tissue (McCarter, unpublished). It appears, therefore, that in the analysis of these tissues, any interference by phosphorus in the determination of total sulphur as benzidine sulphate is likely to be small.

Measurement of ^{35}S The following experiments were made to gain information about the efficiency of the procedure used for separating radioactive sulphate as its benzidine salt.

Four 5 mg samples of radioactive mustard gas were oxidized by the Carius method and the sulphate was precipitated as the benzidine salt. In two of these experiments 1 ml of benzidine reagent was used, and in the other two experiments 2 ml. The precipitates were separated and washed in the usual way and their ^{35}S contents were measured. The supernatant liquids obtained after separating and washing the precipitates were collected, however, instead of being discarded. The two supernatant liquids from each precipitate were combined, concentrated and transferred to a Petri dish. The concentrate was then evaporated to dryness and the radioactivity of the residue was measured. Four similar experiments in which rat skin or liver was oxidized together with the mustard gas were also made.

The results (Table 4) show that the fraction of the radioactive sulphate which was not separated in the benzidine precipitate was negligible, whether the volume of benzidine reagent used was 1 or 2 ml. The results also show that the residue from the oxidation of skin or liver did not interfere with the precipitation of the radioactive sulphate.

Table 4 *Completeness of separation of radioactive sulphate as its benzidine salt*

Material oxidized with 5 mg radioactive mustard gas	Vol of benzidine reagent (ml)	Unseparated ^{35}S (% of ^{35}S separated)
—	1	<0.3
—	1	0.4
—	2	0.3
—	2	0.5
Skin (0.363 g)	1	<0.3
Liver (0.406 g)	1	0.3
Skin (0.343 g)	2	<0.3
Liver (0.369 g)	2	<0.3

Experiments were conducted in which the recovery of ^{35}S was determined when rat tissues (skin, leg muscle, liver, blood) and excreta in amounts similar to those analyzed in tracer studies were oxidized together with weighed amounts of radioactive mustard gas. Four analyses were carried out with each kind of biological material and the results obtained are given in Table 5.

Table 5 *Recovery of ^{35}S added in the form of mustard gas to rat tissues and excreta*

Material	Radioactive mustard gas (mg)	Recovery of ^{35}S (% mean \pm s.d.)
Skin (0.298–0.316 g)	5.06–5.26	98 \pm 1.3
Leg muscle (0.300–0.324 g)	5.06–5.16	100 \pm 2.6
Liver (0.295–0.305 g)	5.07–5.15	99 \pm 2.6
Blood (0.20 ml)	4.84–5.12	100 \pm 2.0
Faeces (0.199–0.297 g)	4.99–5.21	95 \pm 2.7
Urine (0.20 ml)	5.07–5.21	101 \pm 2.7

In order to determine the recovery of ^{35}S when a carcass was analyzed, two experiments were carried out in each of which a rat weighing 165 g was killed, 5 mg of radioactive mustard gas were applied to its skin, and the analysis was completed in the manner described earlier in the paper, the recoveries of ^{35}S were 98 and 96 %

DISCUSSION

The Carius oxidation-benzidine sulphate method is well suited to the routine measurement of ^{35}S in biological material, for the operations involved are simple, require little time, and are such that they can be conducted simultaneously with groups of analyses. A possible objection to the Carius procedure, namely that loss of material is liable to occur as a result of explosion during the oxidation process, is of no significance, for under the experimental conditions described herein, the explosion of a bomb is a very rare occurrence. The benzidine sulphate precipitate can readily be collected in a uniform manner, and although the procedure employed in the present work for preparing the precipitate for radioactivity measurement was designed for use with an ionization chamber apparatus, it can easily be adapted to meet the requirements of other forms of apparatus suitable for the measurement of the radiation from ^{35}S .

The method is applicable to the gravimetric determination of sulphur in a variety of organic compounds (see Table 3). This, in itself, is insufficient, however, to justify the conclusion that the method permits the quantitative recovery of ^{35}S present in such compounds. The weight of the final precipitate in a gravimetric analysis may correspond to the calculated weight as a result of a cancellation of errors, e.g. incomplete precipitation may be offset by occlusion. This possibility can be excluded, however, since it has been found that no significant amount of ^{35}S remains behind after separation of the benzidine sulphate (Table 4). This finding, taken in conjunction with the results of the gravimetric analysis of mustard gas, shows that with the present method the separation of the ^{35}S of radioactive

mustard gas as benzidine sulphate is essentially quantitative. Under these conditions radioactive mustard gas can be used as a source of ^{35}S in studies of the accuracy of the method as a means of measuring ^{35}S in biological material. This was done by comparing the amounts of ^{35}S found when samples of a given specimen of radioactive mustard gas were analyzed alone, and when they were analyzed in the presence of biological material. The results reported in Table 5 show that in a total of twenty-four analyses in which such experiments were carried out with six different kinds of biological material, the mean recovery of ^{35}S was 99 %, and the standard deviation of a single measurement from the mean was 2.8 %. The destructive oxidation of mustard gas does not occur readily, and this fact, when considered together with the findings described above, suggests that the present method is suitable for the measurement of ^{35}S in a variety of biological materials.

SUMMARY

1. A method for the measurement of radioactive sulphur (^{35}S) in biological material is described. A sample of the material is oxidized by the Carius method and the sulphate is precipitated as benzidine sulphate. The radioactivity of the benzidine sulphate is measured in an ionization chamber operated in conjunction with an FP-54 valve amplifying system and a galvanometer, and is compared with the activities of samples of benzidine sulphate prepared from the parent radioactive specimen.

2. When mustard gas prepared from radioactive sulphur was analyzed in the presence of biological material (skin, leg muscle, liver, blood, faeces, or urine) by this method, the mean recovery of ^{35}S was 99 %, and the standard deviation of a single measurement from the mean was 2.8 %.

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A Method for the Colorimetric Micro-estimation of Thymine

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Thymine is the distinctive pyrimidine of the nuclear deoxyribosenucleic acid. A sensitive method for its estimation would be of service in the analysis of nucleic acid, in conjunction with other analytical techniques. Thymine can be distinguished from uracil or cytosine by the stable red colour which is produced when it is coupled with the diazo reagent of Koessler & Hanke (1919) in a solution made alkaline with sodium carbonate and afterwards reduced with hydroxylamine in presence of sodium hydroxide (Hunter, 1936). This reaction has been examined as regards sensitivity, specificity and suitability for quantitative work.

EXPERIMENTAL

Micro estimation of pure thymine

Reagents and procedure Slight modifications were made of Hunter's (1936) procedure. The *diazo reagent* used was that of Koessler & Hanke, viz (i) 1.5 ml sulphamic acid solution (4.5 g in 45 ml conc HCl, made up to 500 ml with water), and (ii) 1.5 ml NaNO_2 solution (25 g in 500 ml water). Solutions (i) and (ii) are quickly mixed and cooled, after 5 min, 6 ml NaNO_2 solution are added and the solution made up to 50 ml. It is kept cold in ice water, and the reagent used not less than 20 min. after making the final dilution. If kept cold it is stable for 24 hr and can conveniently be prepared daily from the stock solutions.

Coupling was performed at 20° and allowed to proceed for exactly 20 min, after which the NaOH was added, followed by hydroxylamine. During the coupling, the tubes should be immersed in a water bath at the prescribed temperature. The extension of time as compared with Hunter's qualitative test (5 min) gives a more intense colour, but no extra tint in similarly conducted blank tests in which distilled water replaces the thymine solution. It is advisable to use stoppered vessels to exclude NH_3 or acid fumes, and to mix the solutions quickly and thoroughly at all stages.

Intensity and stability of the colour was measured by means of a Spekker absorptiometer, using the following amounts of reactants, mixed in the order given: Na_2CO_3 (2.0 ml, 2.4 (w/v) % solution of anhydrous salt), distilled water (2.0 ml), diazo reagent (2.0 ml), thymine solution (2.0 ml containing 0.0125–0.100 mg/ml), NaOH (2.0 ml of 3N), hydroxylamine hydrochloride (0.2 ml of 20% w/v).

Colour values were read using a blue filter (Chance OB2). Beer's law was obeyed up to thymine concentrations of 0.1 mg/ml. Curves have been made with specimens of thymine kindly given by Genatosan Ltd (Loughborough) and Schwarz Laboratories Inc (New York). The samples gave identical readings.

The time of coupling must be measured exactly and the temperature should not vary by more than $\pm 0.5^\circ$. If the reaction is carried out at a higher temperature, e.g. 45° , more intense colours are produced even with very dilute solutions, 10 μg in a final volume of 10 ml can be detected with ease, but these conditions are not recommended for quantitative estimations.

Substances reacting under similar conditions (i) When tested by the above technique none of the purines or pyrimidines known to occur in nucleic acids gives the pink colour except thymine. The other bases yield yellow tints so that if relatively large amounts of these substances are present together with thymine, they affect the colour produced. The presence of uracil in the proportion of 0.1 mg to 0.1 mg thymine occasions little error, however, when the prescribed conditions are observed.

(ii) The reaction yields yellow colours of various intensities with amino acids or proteins at concentrations of 0.2 mg/ml. With cystine a tint very similar to the thymine colour is produced.

(iii) Certain aldehydes, including furfural, and decomposition products produced by mild hydrolysis of nucleic acids, produce pink tints so that precautions must be taken to exclude such substances when estimating thymine. In these circumstances it was not found possible to apply the method directly to hydrolysates of nucleic acid.

Comparison with the method of Harkins & Johnson (1929) These writers described a test for thymine in which the

acetol obtained by refluxing brominated thymine with $\text{Ba}(\text{OH})_2$ is distilled and combined with a reagent produced by heating o nitrobenzaldehyde with FeSO_4 in presence of concentrated NH_3 followed by steam distillation. After acidifying the mixture with HCl and then making alkaline with NaHCO_3 , a blue fluorescence results which is more intense when viewed in the mercury arc light.

I have confirmed the statement that, by this technique, 1 mg thymine can be detected if the volume of the completed test is reduced to 1 ml., but experiments showed that the Hunter (1936) test was much more delicate. Also the 'diazo coupling' method requires only simple apparatus and is much more convenient for routine analyses.

Application of the method to nucleic acid

(a) Technique

The Hunter (1936) reaction cannot be applied directly to a solution of hydrolyzed nucleic acid, since certain reaction products, particularly from ribonucleic acid, also produce red coloured compounds. Efforts to eliminate these, or to extract the thymine quantitatively with various solvents, were not successful. It was, however, found possible to apply the reaction after separating the pyrimidine as a silver compound. The method was based partly on the technique of Levene (1922) for separation of purines, and partly on that of Barnes & Schoenheimer (1943) for the isolation of thymine from thymus nucleic acid. Levene showed that, when HCl gas is passed freely for some time into a suspension of nucleic acid in methanol, heat is liberated, the nucleic acid dissolves and the purines are precipitated. After standing in the cold overnight, they can be removed by centrifugation. Barnes & Schoenheimer evaporated such a supernatant from the purine precipitate to a small volume, hydrolyzed the products by 20% HCl in a sealed tube, and isolated some crystalline thymine.

In the present work the hydrolysate from 50 mg of deoxyribonucleic acid was evaporated to dryness under reduced pressure on the water bath and free HCl removed as thoroughly as possible. The residue was dissolved in 3 ml of 25% (v/v) H_2SO_4 , 1.5 ml transferred to a combustion tube (5-6 in long) and heated in a furnace at 175° for 2.5 hr. Sulphates and phosphates were removed by adding hot, saturated $\text{Ba}(\text{OH})_2$ to pH 9 and centrifuging. The precipitate also carried down all coloured carbonized materials. The supernatant liquid was concentrated to about 10 ml, acidified with HNO_3 to pH 6 and 1 ml of 5% (w/v) AgNO_3 added—shown to be a slight excess by testing externally with $\text{Ba}(\text{OH})_2$ solution. The silver-purine compounds, together with any remaining AgCl , were removed on the centrifuge after standing in the cold. The supernatant was then made alkaline to pH 9 with cold $\text{Ba}(\text{OH})_2$, added drop by drop. A flocculent precipitate resulted, consisting of pyrimidine silver salts together with some silver hydroxide, which, on standing, gave a brownish appearance to the precipitate. After chilling overnight, the mixture was centrifuged, the precipitate washed with a few drops of distilled water, heated to 80° with 5 ml of N HCl to decompose the Ag compounds, and, after cooling, the insoluble AgCl was removed by centrifuging. The solution containing the thymine was made up to 10 ml, solid Na_2CO_3 added until effervescence ceased, the volume adjusted to 25 ml, and 2 ml portions were submitted to the modified Hunter test.

(b) Thymine in commercial nucleic acids

(i) *Thymus nucleic acid* Samples of 20 and 50 mg of the material were subjected to the procedure outlined above, and the results compared with the P content as determined by Holman's (1943) method. The latter gave a figure of 7.3% P as compared with the theoretical of 9.2% for the tetrasodium salt of deoxyribonucleic acid, indicating 80% purity.

Colorimetric estimation of thymine showed a content of 0.128 mg in 20 mg of nucleic acid corresponding to 70% of theory, or 90% of the expected amount based on the P content.

(ii) *Yeast nucleic acid* Repeated analyses of such material demonstrated that only a trace of thymine was present, the final colour being tinged only very slightly pink. The negative diphenylamine reaction (Dische, 1930) obtained with the sample supported the assumption that the deoxyribonucleic acid content was very small.

(c) Thymine in nucleic acid samples from fresh calf thymus

These were prepared by a modification of Hammarsten's (1924) method. In order to remove the cytoplasmic constituents the minced tissue was extracted three times with physiological saline which removes a high proportion of the cytoplasmic nucleoprotein without affecting the nuclear nucleoprotein (Mirsky & Pollister, 1946). The isolation of the nucleic acid was then completed as described by Hammarsten (1924). This method, which yields fibrous nucleic acid, was used since it does not require a high speed centrifuge such as must be employed to clarify the highly viscous solutions of nucleoprotein obtained in Mirsky's method of extraction with M NaCl solution.

The P content of the product was 7.2%, which agreed closely with the value of 7.09% obtained by Hammarsten (1924), but the colour obtained in the quantitative diphenylamine reaction (Dische, 1930, Siebert, 1940) was definitely greater than that given by commercial thymus nucleic acid. When solutions of the same P content were compared, a ratio of 1.13:1.00 was found. The thymine content was 7.05%. These figures suggest that the material was probably free from ribonucleic acid but still retained some impurity. In order to remove this a solution in 2N NaOH was warmed on a water bath for 1 hr at 70° and rendered faintly acid with acetic acid. The precipitate was dissolved in 0.1N NaOH , reprecipitated from ethanol, washed several times with ethanol ether and dried in a desiccator to constant weight.

Portions on analysis gave the following values: (i) P content 8.8% (cf. theoretical 9.18% and for commercial specimen 7.57%), (ii) Dische diphenylamine reaction colour relative to an equivalent solution of commercial nucleic acid was 1.25:1.00, (iii) N content 15.2% (theory 15.6%), (iv) thymine content 9.0% (theory 9.3%), (v) as a further check on the deoxyribose content compared with the commercial specimen, the osymer reaction (Stumpf, 1947) was carried out, yielding a relative colour value of 1.20:1.00.

The material thus appears to have been 96% deoxyribonucleic acid, and the method for thymine estimation, which has been described, is satisfactory for applying to nucleic acid in quantities as small as 20 mg.

Further work is being done to compare the thymine content of deoxyribonucleic acid isolated from animal tumours with that derived from normal tissues.

SUMMARY

1 A colorimetric method is described for the quantitative estimation of thymine which is suitable for quantities between 50 and 200 μg in 2 ml of solution

2 The application of this method to the estimation of thymine in 50 mg samples of deoxyribose-nucleic acid is reported

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Anaerobic Leakage of Potassium from Brain

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Interruption of blood supply to tissues and organs is one of the main causes of organic disease. Characteristic histological changes follow such vascular occlusion, and it seems likely that these have their origin in chemical and metabolic disturbances produced by deprivation of oxygen and food substances from the tissue cells. The present communication describes a study of some of the chemical events taking place when the supply of oxygen and glucose to the cells of the brain is interrupted. These two deficiencies are probably the immediate determinants in the dislocation of cerebral function following vascular accidents, and thus may be regarded as the primary factors in ischaemic damage.

The intracellular concentration of potassium is many times greater than that outside the cells. Danowski (1941) showed that this intracellular potassium diffuses out of red blood cells when the blood sugar is used up, or when glycolysis is inhibited by fluoride. Harris (1941) reported similar results. Dickens & Greville (1933) found that brief anaerobic incubation of slices of cerebral cortex, without substrate, causes inhibition of glycolysis, and Macfarlane & Weil-Malherbe (1941) ascertained that pyrophosphate is destroyed at the same time. Colfer & Essex (1947) observed a loss of potassium from brain during convulsions. Potassium ions are known to cause intense aerobic stimulation and anaerobic inhibition of the glycolytic metabolism of cerebral cortex (Ashford & Dixon, 1935; Dickens & Greville, 1935). For these reasons the present work was designed to find out if leakage of potassium also

occurs from brain when deprived of oxygen and glucose. In this tissue a rise in the external concentration of potassium might produce profound metabolic changes in neighbouring areas outside the initial region of ischaemia as well as affecting radically the internal milieu of the cells primarily affected.

METHODS

Slices of rabbit cerebral cortex were suspended in bicarbonate Ringer solution without glucose made up according to Dickens & Greville (1935) and previously saturated with N_2 containing 5% CO_2 . After washing in this solution, the slices were transferred to Warburg manometer cups containing 1.5 ml of fluid. In general, half the slices were incubated in bicarbonate Ringer without substrate, and the remaining slices in the same volume of the same salt solution containing in addition 0.2% glucose. In some experiments half the slices were incubated in this glucose Ringer, and the remainder in the same glucose Ringer containing 0.05% NaF . In a few experiments three samples of the slices were used, one without substrate, one with glucose and one with glucose and fluoride. The manometers were filled at room temperature with N_2 containing 5% CO_2 , the gas being passed through for 3–5 min.

Potassium estimations were made as follows. Practically the whole of the 1.5 ml of fluid remaining in the manometer cups after incubation at 38° was pipetted away from the slices into centrifuge tubes using Pasteur pipettes. This fluid was then centrifuged at high speed for 15 min and 1 ml of the clear supernatant fluid was pipetted off into Pyrex crucibles and evaporated to dryness in an oven at $100\text{--}120^\circ$. Conc HNO_3 (0.5 ml) was then added to each, and again the crucibles were heated to dryness. The crucibles were then incinerated for 3 hr in an electric muffle furnace at $400\text{--}450^\circ$, to destroy ammonium salts.

The colourless residues were extracted twice with 0.5 ml portions of 0.1N HCl and twice with 0.5 ml portions of distilled water. During extraction the crucibles were briefly heated, each time on a hot plate, till steaming commenced. The four washings from each crucible were transferred, using Pasteur pipettes, to numbered graduated centrifuge tubes and each made up to 2 ml (a little fluid is lost by evaporation during the heating). Sodium cobaltinitrite solution (1 ml, see King, 1946) was added to each tube, and after standing for 45 min the precipitated potassium cobaltinitrite was separated by centrifugation. After draining and washing twice with 70% ethanol, the cobalt in the precipitate was estimated by the method of Jacobs & Hoffman (1931) using the colour reaction with choline chloride and $\text{Na}_2\text{Fe}(\text{CN})_6$. The volume at the final stage was made up to 9 ml, and the colours were compared with similarly treated standard K and standard Co solutions in an Evans photoelectric colorimeter, as designed by King (1946), using an Ilford tricolor red filter as suggested by this author. A sample of the Ringer solution used in the experiment was included in each batch of estimations as a check on recovery following incineration. The recovery of K from this bicarbonate saline was always at least 90 and often 100% (see Table 1). Equivalent amounts of KCl alone cannot be estimated in this way as recovery is extremely poor, presumably owing to unshielded K salts attacking the Pyrex crucibles above 400°. In the ashed bicarbonate Ringer, on the other hand, only a minute fraction of the contained K salts probably comes into sufficiently intimate contact with the Pyrex to cause appreciable loss. It will be evident from the figures given that the recovery of K contained in the incinerated bicarbonate saline is practically complete using the method described above.

Anaerobic glycolysis (Q_L^N) was measured manometrically. Readings were taken every 10 min and the values of Q_L^N were calculated over the first 0.5 hr.

Considerable uniformity in the values of anaerobic glycolysis was shown with different specimens of rabbit brain cortex. Furthermore, glycolysis was fairly constant throughout incubation, only falling by 14% (average of 17 experiments) over 40 min. This constancy contrasts with observations of previous workers using rat brain. For the sake of brevity protocols have had to be omitted.

RESULTS

Table 1 summarizes the results of estimations of potassium in Ringer solution before and after incubation with brain slices for 1 hr. The mean dry weights of tissue used are also shown. Considerable increase in the potassium concentration of the fluid surrounding the tissue was observed, when the medium was devoid of glucose, or contained fluoride as well as glucose. With glucose alone, on the other hand, this increase was eliminated and was generally replaced by slight diminution in the potassium concentration.

A clearer picture of these results is given in Table 2 by evaluating the amounts of potassium liberated or absorbed per unit dry weight of tissue. Mean values for anaerobic glycolysis are also shown. The amount of potassium liberated or absorbed is expressed in μl , on the basis of $1 \mu\text{equiv K} = 11.2 \mu\text{l}$. The number of μl of potassium liberated or absorbed/mg dry wt of tissue/hr gives values of convenient order. A positive value signifies liberation of potassium and a negative value a corresponding absorption. Owing to stringency of space individual values have had to be omitted. In every case except one the results obtained refer to parallel experiments under at least two of the conditions studied with the tissue of each of the rabbits used.

From Table 2 we see that, in the absence of glucose, potassium was in every case liberated into the fluid surrounding the brain tissue, at an average rate of $2.2 \mu\text{l/mg/hr}$. The leakage of potassium was suppressed by glucose and was usually replaced by a small net absorption of potassium ($0.4 \mu\text{l/mg/hr}$). In the presence of glucose the anaerobic glycolysis was high ($Q_L^N = 28 \pm 3$), but in the absence of glucose very small ($Q_L^N = 2 \pm 1$). It appears that when glycolysis can take place the amount of

Table 1 Potassium leakage from brain tissue

(Showing K concentration in Ringer before and after anaerobic incubation for 1 hr at 38° with slices of rabbit-brain cortex. The slices were suspended in 1.5 ml of fluid. Observations on the tissues of 22 rabbits are included.)

		Initial Ringer solution	Initial Ringer after incineration	Ringer after incubation with brain slices in the presence of		
				Glucose 0.2%	No substrate	Glucose 0.2%, NaF 0.05%
K concentration m equiv./l	Mean	2.45	2.37	2.08	3.86	3.62
	Range	2.3-2.6	2.1-2.6	1.7-2.6	3.1-4.9	3.1-3.9
	S.D.	0.11	0.14	0.26	0.48	0.23
	S.E. of mean	0.03	0.03	0.06	0.12	0.07
Dry weight of tissue used (mg.)	Mean	—	—	13.48	11.43	11.22
	Range	—	—	8.1-20.2	7.7-17.3	8.1-16.3
	S.D.	—	—	3.51	2.99	3.15
No. of determinations on initial solutions	—	13	16	—	—	—
No. of rabbits from which tissue was used	—	—	—	21	16	10

Table 2 Potassium leakage from brain in terms of dry weight

(K liberation into the fluid bathing the brain slices during incubation for 1 hr at 38° is expressed as μl of K/mg dry wt of tissue. Positive values indicate liberation of K, negative values indicate absorption. $1 \mu\text{equiv}$ of K = $11.2 \mu\text{l}$. The anaerobic glycolysis is also shown. The figures are from the same series of experiments on the tissues of 22 rabbits referred to in Table 1.)

	μl K/mg dry wt/hr			$Q_L^{N_2}$		
	Glucose 0.2%	No substrate	Glucose 0.2%, NaF 0.05%	Glucose 0.2%	No substrate	Glucose 0.2%, NaF 0.05%
Mean	-0.41	+2.24	+1.89	27.7	1.6	1.9
Range	-1.0 to +0.4	+1.7 to +3.2	+1.2 to +2.5	23-32	0-4	1-3
S.D.	0.36	0.38	0.42	2.6	1.0	0.9
S.E. of mean	0.08	0.10	0.13	0.6	0.3	0.3
No. of rabbits	21	16	10	22	15	10

potassium leaving the cells was more than compensated by increased entry, so that there was usually no net loss of potassium from the cells into the surrounding fluid, and often there was an uptake from the fluid by the cells. In the absence of glycolyzable substrate, however, there was always a highly significant loss of potassium from the cells into the surrounding fluid.

Table 2 also shows that glycolysis was practically eliminated by fluoride ($Q_L^{N_2} = 2 \pm 1$). Danowski (1941) found that the addition of fluoride produced leakage of potassium from human red cells even when blood sugar was present. The results of Table 2 amply confirm this for rabbit brain. When glycolysis was suppressed by fluoride, potassium leaked out into the surrounding fluid just as in the absence of glucose at the rate of $1.89 \mu\text{l}/\text{mg}$ dry wt/hr. In each parallel experiment using glucose alone this leakage was prevented and was often replaced by the actual removal of potassium from the fluid. The suppression of potassium leakage was not due to the mere static presence of glucose, but to its dynamic utilization in supplying energy to the glycolyzing cell, since when glycolysis was inhibited leakage again became evident.

In the absence of glycolyzable substrate appreciable potassium leakage occurred even in half an hour. This is apparent from Table 3 which records observations with brain tissue of two rabbits incubated for only half an hour, in these two experiments about $1.0 \mu\text{l}$ K/mg dry weight was liberated in half an hour. Approximately twice this amount (average $2.2 \mu\text{l}$) leaked out in 1 hr (Table 2). In Table 3 the effect of glycolyzable substrate in curbing leakage and promoting uptake is also evident.

Table 3 Potassium accumulation/mg dry weight in fluid incubated with brain slices for 0.5 hr

Rabbit no	μl K liberated/mg dry wt/0.5 hr		
	Glucose	No substrate	Glucose fluoride
10	-1.0	+1.0	+1.1
11	-1.0	+1.0	—

Some potassium leakage may even occur while setting up the experiments at room temperature. Thus in three cases Ringer solution without substrate or with glucose-fluoride was treated with brain slices, and equilibrated with nitrogen/carbon dioxide under the same conditions as in the actual experiments up to the point of the start of incubation. Then, instead of being placed in the bath at 38°, the fluid was removed from the tissue slices for analysis without incubation. In two cases without substrate the potassium concentration of the fluid had risen so that 0.9 and 0.6 μl of potassium were liberated per mg dry weight. In the third case with glucose-fluoride, however, no increase was detected. After incubation for 1 hr, tissue from these three rabbits liberated 2.0-2.2 μl of K.

When both glucose and oxygen were supplied to the cells (just as with glucose alone) no net leakage of potassium occurred, and the uptake of potassium from the fluid by the tissue is apparently even more definite than with glucose alone. Thus with tissue from two of the rabbits parallel experiments were set up with glucose and oxygen/carbon dioxide as well as the anaerobic experiments recorded in Table 2. In one case with glucose alone the rate of disappearance of potassium was $-0.6 \mu\text{l}/\text{mg}$ dry wt/hr, but with glucose and oxygen this was $-1.0 \mu\text{l}/\text{mg}$ dry wt/hr, while in the absence of both glucose and oxygen, there was a net accumulation of potassium at the rate of $+1.7 \mu\text{l}/\text{mg}$ dry wt/hr. Similarly, in the other case, with glucose alone, the disappearance of potassium was $-0.6 \mu\text{l}/\text{mg}$ dry wt/hr, but with glucose and oxygen this was $-1.2 \mu\text{l}/\text{mg}$ dry wt/hr, whereas the anaerobic accumulation with glucose and fluoride was $+2.1 \mu\text{l}/\text{mg}$ dry wt/hr. It thus appears that glucose and oxygen checked the leakage and promoted uptake of potassium even more effectively than glucose alone.

How far potassium leakage is accompanied by tissue disintegration is uncertain. Lutwak-Mann (1947) found tissue disintegration of gastric mucosa to be promoted by anaerobic substrate deprivation. In the present experiments with brain also more

debris was usually formed in the absence of substrate than when glucose was present. In one experiment an exceptionally large amount of debris was formed without substrate, this was much more marked than with glucose and even more marked than with glucose-fluoride. However, the leakage of potassium was no greater without substrate than with glucose-fluoride. This indicates that potassium leakage is probably not mainly occasioned by mere tissue disintegration, but the two phenomena may well progress together. In the later experiments of this series the spun debris was included in the dry weight determinations.

DISCUSSION

We may conclude from the results recorded in Tables 1-3 that when both the supply of glucose and oxygen to the brain cells is cut off there is considerable leakage of potassium into the extracellular fluid. This loss of potassium may be one of the basic factors responsible for ischaemic injury to cells, as potassium is doubtless an essential intracellular constituent on whose presence in high concentration many enzymic processes may depend. Furthermore, in the case of brain, it is possible that the loss of potassium from damaged cells may have other consequences since in brain potassium ions have such remarkable effects on metabolism. The irritation and paralysis associated with vascular

accidents may thus depend in part on these metabolic effects produced by potassium ions which have emerged from the primary focus of ischaemic damage out into adjacent regions, as well as on the escape of essential intracellular constituents (such as potassium) out of the initially injured tissue.

SUMMARY

1 During anaerobic metabolism in the presence of glucose there was no increase in the concentration of potassium in the fluid bathing slices of cerebral cortex. In some cases there was a decrease.

2 Under the same conditions, but in the absence of glucose, there was always a significant rise in the potassium concentration of the fluid surrounding the brain slices.

3 The action of glucose in preventing this loss of potassium is dynamic: it depends on the active utilization of glucose by the glycolyzing cell. Where glycolysis was inhibited by fluoride potassium leaked into the environmental fluid just as in the absence of glucose. Brain thus resembles red blood cells for which Danowski (1941) has described a similar effect.

4 The significance of these findings on the nature of ischaemic damage is briefly considered.

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The Activation of Phosphoglucomutase by Metal Ions

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The existence of glucose 1-phosphate and its conversion to glucose-6-phosphate in muscle were first described by Cori & Cori (1936). Kendal & Stickland (1938) claimed that Mg^{++} and fructofuranose 1,6-diphosphate were both essential for this conversion, but Cori, Colowick & Cori (1938) stated that 'the contention of Kendal & Stickland that hexosediphosphate acts as a coenzyme and is essential for the reaction is not substantiated by our findings'. They found, on the other hand, that

Mn^{++} and Co^{++} are more efficient activators than Mg^{++} , and that the enzyme (named by them phosphoglucomutase) shows a large residual activity in the absence of any added metal. This has led to the view, expressed typically by Sumner & Somers (1947), that 'phosphoglucomutase requires no coenzyme'.

The present paper deals with some of the effects of metallic ions on the activity of phosphoglucomutase. In a later publication the author hopes to

describe the role of fructofuranose 1 6 diphosphate (*HDP*), and to reconcile the conflicting statements of Cori *et al* (1938) and Kendal & Stickland (1938) For the present it is enough to reassert that *HDP* is essential for maximal activity of phosphoglucomutase, as will be seen later in Table 1

METHODS

Phosphoglucomutase In most of the experiments the enzyme was partially purified by the method of Colowick & Sutherland (1942), but similar results can be obtained equally well in a simple dialyzed muscle extract

Glucose 1 phosphate This was prepared by a method substantially the same as that of Sumner & Somers (1943), and was used as the K salt

Salts of metals The salts used were A.R., where this quality was available (e.g. potash alum, chrome alum and lead acetate), in the other cases ordinary commercial samples were used without special purification

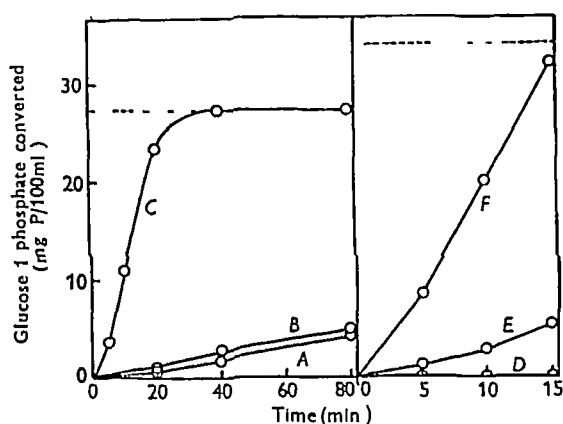


Fig 1 The course of the conversion of glucose 1 phosphate to glucose 6 phosphate. Curve A, $\text{Al}^{+++} + \text{HDP}$, curve B, $\text{Mg}^{++} + \text{HDP}$, curve C, $\text{Al}^{+++} + \text{Mg}^{++} + \text{HDP}$, curve D, $\text{Mg}^{++} + \text{HDP}$, curve E, $\text{Mn}^{+++} + \text{HDP}$ or $\text{Cr}^{+++} + \text{Mn}^{+++} + \text{HDP}$, curve F, $\text{Cr}^{+++} + \text{Mg}^{++} + \text{HDP}$. The dotted lines indicate the amount of conversion at equilibrium. The concentration of *HDP* was 0.0003M, and that of the metals was optimal (see text)

Measurement of the course of reaction The reaction was followed by determining the residual glucose 1 phosphate by 5 min hydrolysis in N HCl at 100° . The reaction mixtures contained the substrate, etc., at the following final concentrations, unless otherwise stated: glucose 1 phosphate, 0.01M, Na phosphate buffer, pH 7.5, 0.005M (approx), *HDP*, 0.0003M. The various metals and their concentration will be given in the text and tables. For the sake of uniformity, concentrations are expressed in terms of the 'molarity' of cation (e.g. Cr^{+++}) and not of the actual salt used (e.g. $\text{Cr}_2(\text{SO}_4)_3$). The reaction mixtures were made up in lots of 1.0 or 2.0 ml in 6×0.5 in test tubes, and were placed in the water bath at 38° for some 5 min before the addition of the enzyme solution (0.1 or 0.2 ml at a pre-determined dilution). The reaction was stopped, usually after 15 min, by the addition of 3 vol of 4% trichloroacetic acid and the inorganic PO_4 determined in samples of the filtrate before and after 5 min acid hydrolysis at 100°

(Fiske & Subbarow, 1925). When the time course of the reaction was to be followed, larger volumes of mixture were made, and 1.5 or 2.0 ml samples withdrawn from each into trichloroacetic acid at zero time and after the required intervals.

The use of a single point to measure relative reaction velocities was justified by the observation that the reaction is almost linear until the equilibrium point is nearly reached. A slight lag in the first quarter of the reaction was sometimes noticed (Fig 1). To obtain the greatest possible accuracy, the concentration of the enzyme was always adjusted so that, in the fastest reaction of any series, some 75% of the conversion had taken place in the time allowed (15 min). Finally, the most important points were checked by following the course of the reaction to the equilibrium point.

RESULTS

The basic observation is that maximal activation of phosphoglucomutase is achieved only with a combination of three components, viz. two metals and *HDP* (Table 1). Of these three components, the present work is concerned only with the two metals, and, in all the experiments that follow, it may be assumed that *HDP* is present at a concentration of 0.0003M unless the contrary is stated. A wide range of metals has been tested for their power to take the place of either Mg^{++} or Al^{+++} in the system shown in Table 1, and these will be dealt with in turn.

Table 1 The indispensability of three components for full phosphoglucomutase activity

(Reaction mixture as described in text. Concentrations of additions: Mg^{++} , 0.003M, Al^{+++} , 0.0005M, *HDP*, 0.0003M)

Additions	Glucose 1 phosphate converted in 15 min (mg P/100 ml)		
	Exp 1	Exp 2	Exp 3
$\text{Mg}^{++} + \text{HDP}$	1.1	0.0	0.0
$\text{Al}^{+++} + \text{HDP}$	3.1	1.3	0.0
$\text{Mg}^{++} + \text{Al}^{+++}$	1.6	1.7	0.7
$\text{Mg}^{++} + \text{Al}^{+++} + \text{HDP}$	27.7	22.6	11.0

Magnesium The indispensability of Mg^{++} was demonstrated by the use of a test system containing substrate, enzyme, Al^{+++} and *HDP*. The addition of other metals to this system showed that only Mg^{++} led to the appearance of any considerable activity (Table 2). Cr^{+++} is not included in this table, as it is a special case which will be considered later. The concentrations used, except in the case of Mg^{++} , were 0.002 and 0.0002M, and it is most unlikely that any activity should exist which would not be detected at one or other of these concentrations. Mg^{++} was tested at 0.004M, the concentration shown to be optimal for glycolysis by Lohmann (1931). Cori *et al* (1938) observed an ill-defined optimum for phosphoglucomutase at c. 0.005–0.01M.

Of special interest are the negative results with Mn^{++} and Co^{++} , these will call for further comment later

Table 2 *The irreplaceability of Mg^{++}*

(Reaction mixture and conditions of experiment as described in the text. Concentrations Al^{+++} (0.005M) and *HDP* (0.0003M) present throughout, Mg^{++} , 0.003M, other metals, 0.002M. The other metals were also tested at 0.0002M, with similar negative results. Other metals tested, at 0.002 and 0.0002M, with completely negative results, were Be^{++} , Ca^{++} , Zn^{++} , Pb^{++} , Hg^{++} , UO_2^{++} , La^{+++} , Bi^{+++} , Zr^{++++} , Ce^{+++} .)

Addition	Glucose 1 phosphate converted in 15 min (mg P/100 ml)		
	1st series	2nd series	3rd series
None	2.7	0.1	0.2
Mg^{++}	26.4	21.7	12.3
Fe^{+++}	3.3	—	—
Mn^{++}	3.2	—	—
Sr^{++}	—	2.3	—
Ba^{++}	—	1.7	—
Cu^{++}	—	2.0	—
VO^{++}	—	—	1.1
Ni^{++}	—	—	2.5
Co^{++}	—	—	1.0
Ti^{++++}	—	—	1.1

The relationship between concentration of Mg^{++} and degree of activity is shown in Fig. 2. The optimum, 0.003M, agreed closely with that found

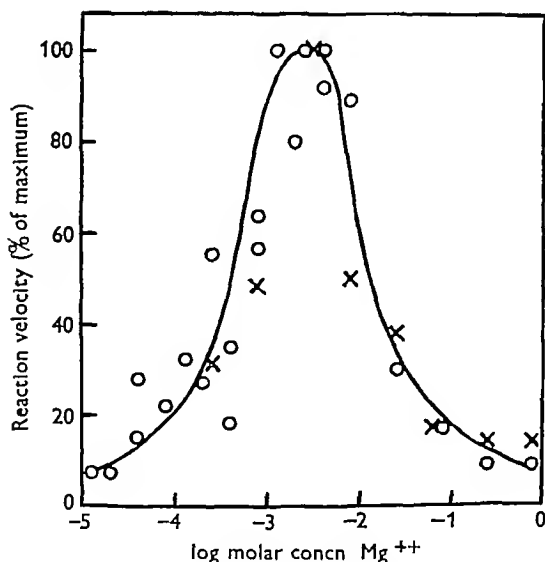


Fig. 2 The relationship between concentration of Mg^{++} and activity of phosphoglucomutase. *HDP* (0.0003M) present throughout. Circles in the presence of Al^{+++} (0.0005M), crosses in the presence of Cr^{+++} (0.00002M).

by Lohmann (1931) for the whole glycolytic process, but not so closely with that found by Cori *et al* (1938) for partially activated phosphoglucomutase

It was the same whether the second metal were Al^{+++} or Cr^{+++} , the optimum for Mg^{++} has not been specially determined for any other metals, and Mg^{++} has been used at 0.003M in all other experiments.

The second metal. In a test system containing substrate, enzyme, Mg^{++} and *HDP*, the inclusion of Al^{+++} led to the appearance of a high enzymic activity (Table 1), and this power was shared by a number of other metals, notably Cr^{+++} , Pb^{++} , Fe^{+++} , UO_2^{++} , Ce^{+++} , Ti^{++++} , Be^{++} , etc (Table 3).

Table 3 *Metals which can supplement Mg^{++}*

(Reaction mixture and conditions of experiment as usual. Concentrations Mg^{++} (0.003 or 0.004M) and *HDP* (0.0003M) present throughout, Al^{+++} and other metals, 0.0002M. The other metals were also tested at 0.00002M, without revealing any further activity. Other metals tested, at 0.0002 and 0.00002M, with completely negative results, were Zn^{++} , Ca^{++} , Sr^{++} , Cu^{++} , Hg^{++} , Ni^{++} , VO^{++} , Bi^{+++} , Ag^{+} , and Cd^{++} . The salts used were the sulphates, except those of Be , Ca , Sr , Ba , Hg , Zr , Cs and Sn (chlorides), UO_2 , La and Ag (nitrates), and Pb (acetate).)

Addition	Glucose 1 phosphate converted in 15 min (mg P/100 ml)			
	1st series	2nd series	3rd series	4th series
None	0.7	3.5	2.8	2.3
Al^{+++}	16.2	21.7	12.3	25.6
Mn^{++}	1.8	—	—	—
Be^{++}	13.7	—	—	—
Cr^{+++}	37.6	—	—	—
Fe^{+++}	24.4	—	—	—
Ba^{++}	—	8.4	—	—
Pb^{++}	—	19.0	—	—
UO_2^{++}	—	16.9	—	—
La^{+++}	—	—	5.8	—
Zr^{++++}	—	—	5.3	—
Ti^{++++}	—	—	9.8	—
Ce^{+++}	—	—	10.6	—
Cs^{+}	—	—	—	4.0
Tl^{+}	—	—	—	2.9
Sn^{++}	—	—	—	5.8

With all these metals the activity was found only if Mg^{++} was present at the same time (Table 4). A partial exception to this rule was found in Cr^{+++} , which at relatively high concentrations (of the order of 10^{-4} M) showed some activity in the absence of Mg^{++} (see bottom of Table 4). This point will be considered in rather more detail later.

The relationship between the concentration of the individual metals and the degree of activity produced in the presence of Mg^{++} and *HDP* showed in every case (except that of Cr^{+++}) a fairly sharp optimum. Although each single enzyme preparation gave a smooth and reproducible relationship between activity and concentration of cation, different preparations gave slightly different curves, so it is impossible to be very precise about the affinities of the enzyme for the metals. These points are illus-

Table 4 *The indispensability of Mg^{++}* (Reaction mixture and conditions of experiment as before HDP (0.0003M) was present throughout, Mg^{++} , 0.003M)

'Second metal'	Concentration of 'second metal' ($\mu r \times 10^3$)	Glucose 1-phosphate converted in 15 min (mg P/100 ml)		
		Mg^{++} alone	'Second metal' alone	Both together
Al^{+++}	60	1.1	3.1	27.7
Al^{+++}	40	0.7	0.4	23.4
Be^{++}	23	0.8	0.0	9.8
UO_2^{++}	67	0.0	0.0	9.6
UO_2^{++}	20	1.5	1.1	9.5
Pb^{++}	50	0.0	0.4	20.1
Fe^{+++}	67	0.8	0.0	12.7
Ce^{+++}	40	0.0	0.0	15.8
Cr^{+++}	4	0.0	0.0	24.8
Cr^{+++}	2	1.5	2.0	20.1
Cr^{+++}	1.2	0.5	0.0	19.7
Cr^{+++}	45	0.8	9.0	25.9
Cr^{+++}	12	0.5	15.9	22.3

trated in the case of Al^{+++} in Fig. 3, the other metals (except Cr^{+++}) gave curves of similar shape, whose characteristics are shown numerically in Table 5

a single phosphoglucomutase preparation, might show greater activity if tests were carried out on a wider scale

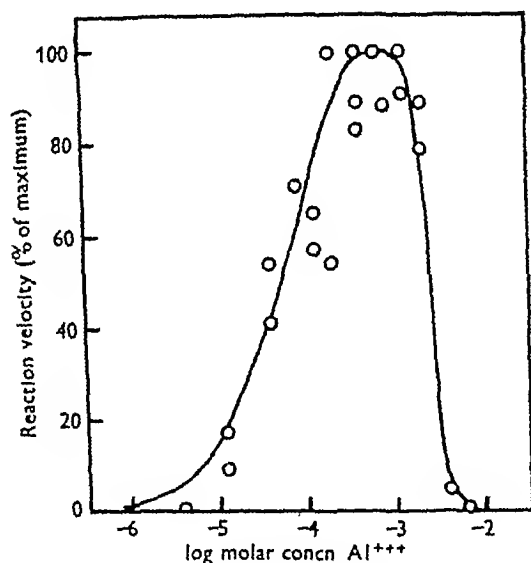


Fig 3 The relationship between concentration of Al^{+++} and activity of phosphoglucomutase HDP (0.0003M) and Mg^{++} (0.003M) present throughout

The maximal activity reached with $Al^{+++} + Mg^{++}$, $Cr^{+++} + Mg^{++}$, and $Pb^{++} + Mg^{++}$, each at their optimal concentrations, was the same. With Fe^{+++} , Fe^{++} , and Ce^{+++} the maximal activity in the presence of Mg^{++} was only slightly lower, being from 70 to 90% of that with $Al^{+++} + Mg^{++}$. The other metals gave results which varied widely from one phosphoglucomutase preparation to another (Table 5). In view of this variability it must be admitted that some of the metals, which in Table 3 showed a small activity (e.g. Ba^{++} , La^{+++} , Zr^{++++} , and Sn^{++}) and which were there tested only with

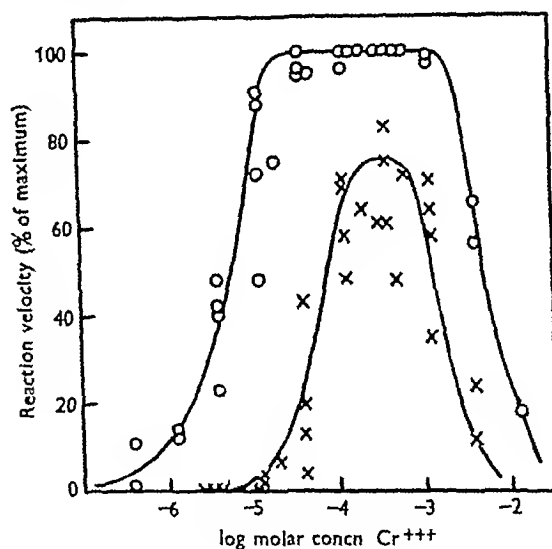


Fig 4 The relationship between concentration of Cr^{+++} and activity of phosphoglucomutase. Circles in the presence of HDP (0.0003M) and Mg^{++} (0.003M), crosses in the presence of HDP (0.0003M) only

Chromium behaved exceptionally in several ways. In the presence of Mg^{++} , full activity equal to that with $Al^{+++} + Mg^{++}$ was found over the whole range of Cr^{++} concentrations from 10^{-5} to 2×10^{-3} M, and half the maximum was reached at 5×10^{-6} M (Fig. 4). This high affinity of the enzyme for Cr^{+++} distinguishes this element from Al^{+++} and all the other metals. A second point of difference was that Cr^{+++} showed a considerable degree of activity in the absence of Mg^{++} . This activity was only some 75% of that observed when Mg^{++} was present too, and was found only at the higher part of the range

Table 5 *The relationships between the concentrations of various metals and the phosphoglucomutase activity produced by them in the presence of $Mg^{++} + HDP$*

Metal	Salt used	Relative activity ($Al^{+++}=100$)	Concentration giving half the maximal activity (M)	Optimal concentration (M)
Al^{+++}	$KAl(SO_4)_2 \cdot 12H_2O$	100	6×10^{-5}	5×10^{-4}
Cr^{+++}	$KCr(SO_4)_2 \cdot 12H_2O$	100	5×10^{-6}	10^{-5} to 2×10^{-3}
Pb^{++}	$Pb(OOCCH_3)_2$	100	3×10^{-5}	4×10^{-4}
Fe^{+++}	$KFe(SO_4)_2 \cdot 12H_2O$	70-80	1×10^{-4}	4×10^{-4}
	$Fe(NO_3)_3 \cdot 9H_2O$			
Fe^{++}	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	70	5×10^{-5}	4×10^{-4}
Co^{+++}	$Co_2(SO_4)_3$	80-90	6×10^{-5}	3×10^{-4}
Ti^{++++}	$Ti(SO_4)_2$	30-80	4×10^{-5}	3×10^{-4}
		(5 enzyme samples)		
		0		
		(1 enzyme sample)		
UO_2^{++}	$UO_2(NO_3)_2 \cdot 6H_2O$	35-80	4×10^{-4}	3×10^{-4}
		(6 enzyme samples)		
		0		
		(2 enzyme samples)		
Be^{++}	$BeCl_2 \cdot 4H_2O$	15-70	1×10^{-4}	5×10^{-4}

of concentrations of Cr^{+++} . There was consequently an appreciable range of Cr^{+++} concentrations over which no activity was seen unless both metals were present together (from 10^{-5} to 4×10^{-5} M). The activity with Cr^{+++} alone showed a sharp maximum at 3×10^{-4} M, half this maximum being reached at about 8×10^{-5} M.

Manganese and cobalt. Cori *et al.* (1938) showed that the activity of phosphoglucomutase is increased by Mg^{++} , Mn^{++} and Co^{++} , the degree of activity produced being roughly the same for all three, and the concentration required for full activation being greater for Mg^{++} than for the other two. They also observed that at lower enzyme concentrations the efficacy of Mg^{++} became less than that of Mn^{++} or Co^{++} . However, at the still lower enzyme concentrations necessitated by the greater activity of the systems dealt with in the present work, the activity with Mg^{++} was negligibly small, or zero (see Tables 4 and 6-8). Under the same conditions the activity with Mn^{++} added alone was considerably greater than that with Mg^{++} alone, but still small compared with that given by $Mg^{++} + Cr^{+++} + HDP$. The ratio

$$\frac{\text{Activity with } Mg^{++} + Cr^{+++} + HDP}{\text{Activity with } Mn^{++}}$$

for eight different enzyme preparations is shown in Table 6, it varied from 5 to 12, with an average of 7.5. Co^{++} , used alone, showed about the same activity as Mn^{++} (Table 8).

In an earlier section it was stated that neither Mn^{++} nor Co^{++} could replace Mg^{++} in the complete system (Table 2). In the experiments to which that table refers, these metals were tested at concentrations of 0.002 and 0.0002 M. The difference in behaviour was so curious that it seemed advisable to test these two metals more thoroughly, and in particular at the optimal concentrations already

established by Cori *et al.* (1938). The results appear in Tables 6-8. It is plain that (a) neither Mn^{++} nor Co^{++} can act in the same way as Mg^{++} in supplementing the action of $Cr^{+++} + HDP$, and (b) they will not supplement the action of any of the other metals which, in co-operation with $Mg^{++} + HDP$, can produce full activity of the enzyme. In fact, many of the metals which, when present with Mg^{++} , stimulate the enzyme, had an inhibitory effect on that activity which is produced by Mn^{++} alone. Another point of interest is that the effect of Mn^{++} is independent of the presence of HDP (see Table 9).

The effect of pH. The need for three activating components was observed equally at all pH values at which the enzyme was active (Table 10). These results were obtained with the use of a mixed phosphate (0.005 M) and veronal (0.005 M) buffer. Veronal at high concentrations inhibited the enzyme (50% inhibition at 0.0125 M), but at 0.005 M the inhibition was only c. 10%, and as the veronal was present in all the reaction mixtures the inhibition can be ignored. Borate is less suitable for buffering phosphoglucomutase, as it also inhibits it completely and at a rather lower concentration (50% inhibition at 0.007 M). The substrate also contributes largely to the buffering of the reaction mixtures used in these experiments, so the appropriate mixtures of glucose 1-phosphate, phosphate and veronal were made up, and their pH's adjusted to the required values by a colorimetric method, before they were measured out into the tubes in which the reaction was to take place. The fact that no difference in behaviour was noted obviated the need to make more precise measurements of the pH values. The enzyme preparation (at pH 7.5) was used at such great dilution that its addition had no effect on the pH of the mixtures.

Table 6 *The failure of Mn^{++} to replace Mg^{++} in the system $Mg^{++} + Cr^{+++} + HDP$*

(Reaction mixture and conditions of experiment as given in the text Concentrations HDP , 0.0003M (present throughout), Mg^{++} , 0.003M, Mn^{++} , 0.00125M, Cr^{+++} , 0.00002 or 0.00004M)

Glucose 1-phosphate converted in 15 min (mg P/100 ml) in the presence of					Ratio of activities $Cr^{+++} + Mg^{++}$
Mg^{++}	Mn^{++}	Cr^{+++}	$Cr^{+++} + Mg^{++}$	$Cr^{+++} + Mn^{++}$	Mn^{++}
1.0	1.4	0.0	17.2	1.9	12.3
0.0	5.3	3.5	32.0	5.4	6.0
1.2	4.6	1.3	22.9	2.4	5.0
0.0	2.0	0.6	13.1	2.5	6.6
0.0	1.9	—	20.7	—	10.9
1.3	4.4	—	22.6	—	5.6
2.8	3.3	—	27.0	—	8.2
1.6	5.7	3.8	31.2	—	5.5

Table 7 *The failure of Mn^{++} to replace Mg^{++} in the system $Mg^{++} + HDP + other metals$*

(Reaction mixtures and conditions of experiment as described in the text Concentrations HDP , 0.0003M (present throughout), Mg^{++} , 0.003M, Mn^{++} , 0.00125M, Al^{+++} , 0.0004M, Pb^{++} , 0.0005M, Fe^{+++} , 0.00033M, UO_2^{++} , 0.00067M, Ce^{+++} , 0.0004M)

Second metal (X)	Glucose 1 phosphate converted in 15 min (mg P/100 ml) in the presence of				
	Mg^{++}	Mn^{++}	X	$Mg^{++} + X$	$Mn^{++} + X$
Al^{+++}	0.8	3.3	2.3	23.2	1.3
Al^{+++}	1.2	4.6	4.6	18.8	0.7
Al^{+++}	0.0	2.0	0.0	12.8	1.5
Fe^{+++}	0.8	3.3	0.0	12.7	2.5
Fe^{+++}	0.0	2.0	1.4	10.1	1.5
Pb^{++}	0.0	2.3	0.4	10.1	0.0
UO_2^{++}	0.0	2.3	0.0	9.6	0.0
Ce^{+++}	0.0	2.3	0.0	15.8	1.6

Table 8 *The failure of Co^{++} to replace Mg^{++} in the system $Mg^{++} + HDP + various metals$*

(Reaction mixture and conditions of experiment as before Concentrations HDP , 0.0003M (present throughout), Cr^{+++} , 0.00002 or 0.00004M, Al^{+++} , 0.0004M, Fe^{+++} , 0.00033M, Pb^{++} , 0.0005M, Ce^{+++} , 0.0004M, Mg^{++} , 0.003M, Co^{++} , 0.0017M)

Second metal (X)	Glucose 1 phosphate converted in 15 min (mg P/100 ml) in the presence of				
	Mg^{++}	Co^{++}	X	$Mg^{++} + X$	$Co^{++} + X$
Cr^{+++}	1.2	5.7	1.3	22.9	7.1
Cr^{+++}	0.0	1.9	0.6	13.1	2.5
Al^{+++}	1.2	5.7	4.6	18.8	1.3
Al^{+++}	0.0	1.9	0.0	12.8	1.1
Fe^{+++}	0.0	1.9	1.4	10.1	0.3
Pb^{++}	1.2	5.5	0.4	23.0	6.7
Ce^{+++}	1.2	5.5	0.0	23.6	4.6

Table 9 *The absence of any effect of HDP on the activity of phosphoglucomutase activated by Mn^{++} alone*

(Reaction mixture and conditions of experiment as before Concentrations Mn^{++} , 0.00125M, HDP , 0.0003M)

Glucose 1 phosphate converted in 15 min (mg P/100 ml)	
HDP absent	HDP present
24.0	24.4
12.0	11.0
6.0	6.5
3.1	2.6

The effect of progressive dilution of the enzyme
Since the activity of phosphoglucomutase with Mg^{++} , Cr^{+++} and HDP is some ten times that previously observed with Mg^{++} or Mn^{++} , it is clear that in order to measure this activity either the time scale of the experiments must be shortened or the enzyme concentration must be reduced. In the present work the method chosen was that of decreasing the enzyme concentration, keeping the time scale constant.

With the object of determining the proper enzyme concentration, a large number of routine tests have been carried out, using serial twofold dilutions of the enzyme, and the results of these show some

features of interest Fig 5 gives a diagrammatic summary of many such experiments At the highest concentrations no activator was needed, with decreasing concentrations this activity rapidly fell off (Fig 5, curve A), but full activity could still be achieved by addition of Mg^{++} or Mn^{++} With a small

Table 10 *The indispensability of two metals at various pH values*

(The preparation of the reaction mixtures and the conditions of experiment are described in the text Concentrations *HDP*, 0.0003M (present throughout), Cr^{+++} , 0.00002M, Mg^{++} , 0.003M)

	Glucose 1 phosphate converted in 15 min (mg P/100 ml)		
	pH 7.5	pH 8.0	pH 8.5
Cr^{+++} alone	1.2	2.8	1.4
Mg^{++} alone	1.9	2.8	0.9
$Cr^{+++} + Mg^{++}$	27.2	25.7	18.7

further dilution of the enzyme, the Mg^{++} activation rapidly disappeared (curve B), while the activation with Mn^{++} fell off more slowly (curve C) At a point where the activity with Mg^{++} had vanished, and that with Mn^{++} had become very small, full activity could still be produced by the addition of Cr^{+++} and Mg^{++} Eventually this activity also abruptly disappeared with further dilution (curve D)

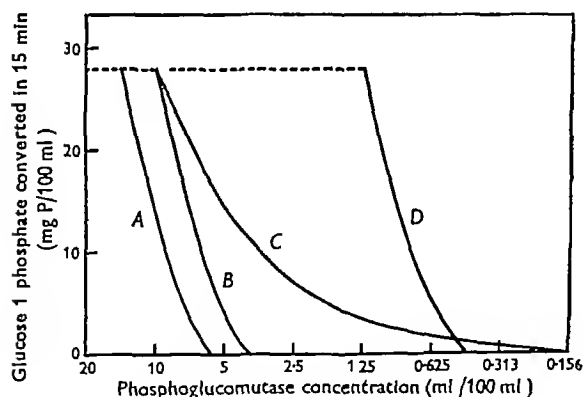


Fig 5 Diagrammatic representation of the effect of dilution of phosphoglucomutase on its activity in the presence of various activators Curve A, no activator, curve B, Mg^{++} , curve C, Mn^{++} , curve D, $Mg^{++} + Cr^{+++} + HDP$ (0.0003M) present throughout

A curious point is that the velocity of reaction was proportional to the enzyme concentration only in the experiments where Mn^{++} was the sole activator (Fig 5, curve C, see also Table 9, where the four results were obtained with successive twofold dilutions of the enzyme) That the reaction velocity should diminish more rapidly than the enzyme concentration might be expected in experiments with no added activator, or in those with Mg^{++} added

alone (curves A and B), but in fact precisely the same relationship was observed when the enzyme was fully activated with Mg^{++} and Cr^{+++} (curve D)

Owing chiefly to variable losses in activity during the heat treatment in the purification of the enzyme, the absolute volume of enzyme required varied considerably from one preparation to another On the other hand, the relative volumes required for measuring the activity in the presence of different combinations of activators were fairly constant The scale of enzyme concentrations shown in Fig 5 is the rough average of those found with a large number of phosphoglucomutase preparations, in which the final volume of the enzyme solution was one fifth of that of the original muscle extract

The absolute activity of phosphoglucomutase preparations Schlamowitz & Greenberg (1948) say that the method of Colowick & Sutherland (1942) gives activities of phosphoglucomutase up to 3300 units/mg of protein, these activities being measured in the presence of 0.00125M Mn^{++} Similar measurements on four samples of phosphoglucomutase in the presence of optimal concentrations of Mg^{++} , Cr^{+++} , and *HDP* gave values of 20,000, 37,000, 26,000 and 11,000 units/mg dry weight, figures which confirm the value for the ratio

$$\frac{\text{Activity with } Mg^{++} + Cr^{+++} + HDP}{\text{Activity with } Mn^{++}}$$

shown in Table 6

DISCUSSION

The chief facts presented in this communication do not call for further discussion at the moment It might, however, be profitable to inquire into the possible physiological implications Previously the most active form of phosphoglucomutase had been that obtained by the addition of Co^{++} , Mn^{++} or Mg^{++} The concentrations of Co^{++} or Mn^{++} required were enormously in excess of those which could occur in animal tissues, so Mg^{++} was accepted as the only physiological activator The present results show that, at a great dilution of the enzyme, Mg^{++} , either alone or in co operation with *HDP*, imparts no activity to the enzyme, and that another metal is required in addition This phenomenon is capable of more than one interpretation, but as suming for the moment that the activation by two metals is of importance *in vivo*, then only Cr^{+++} appears to be worth considering as an actual physiological component of the system, because of the higher concentrations required with all the others Cr^{+++} shows maximal activity at a concentration of 10^{-5} M, or 50 $\mu g/100 g$ It remains to be seen whether a concentration of this order is to be found in animal tissues, the only report in the literature is that of Dutoit & Zbinden (1930), who by spectro

graphic analysis detected traces of Cr^{+++} in all organs, with most in thyroid and spleen

The relationship between the activation by Mn^{++} and that by $\text{Mg}^{++} + \text{Cr}^{+++} + \text{HDP}$ is curious. The variability in the ratio of these two activities (see Table 6) at first indicated that two different enzymes might be concerned, but the absence of any marked change in the ratio during the purification of the enzyme, and the variability in some other properties of the enzyme between one preparation and the next, suggest that this is not so. Further work on the interrelations between the activations by various metals and combinations is proceeding.

SUMMARY

1 The greatest activity of phosphoglucomutase is found to occur only in the simultaneous presence

of three activators, hexosediphosphate, Mg^{++} and a second metal

2 The second metal may be Al^{+++} , Cr^{+++} , Pb^{++} , Fe^{+++} , or Ce^{+++} , some other metals also show smaller activity

3 In this system Mg^{++} cannot be replaced by Mn^{++} or Co^{++} . The maximal activity is some ten times that previously observed with Mn^{++} as the only activator

4 Of all the 'second metals' studied, only Cr^{+++} shows any activity in the absence of Mg^{++} , and that only at relatively high concentrations

5 Consideration of affinities suggests that if this 'two metal' activation has any physiological importance, then Cr^{+++} and Mg^{++} are the metals concerned

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The Effect of the Peroxide Concentration and other Factors on the Decomposition of Hydrogen Peroxide by Catalase

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When catalase is added to hydrogen peroxide there is an initial rapid evolution of oxygen which lasts for about 2 min. After this oxygen is given off at a steady rate which slowly decreases in the course of about an hour. This is not necessarily due to a decrease in the peroxide concentration, since it is quite marked in experiments where there is a large excess of peroxide (Morgulis, Beber & Rabkin, 1926; George, 1947). The first problem in studying the kinetics of the reaction is to determine to what extent destruction of the enzyme is responsible for these changes in the rate as the reaction proceeds.

The results of some of the early investigations are difficult to interpret because only the total amount of oxygen evolved from a given amount of catalase and peroxide is recorded, so that the initial rapid

reaction and the steady evolution cannot be distinguished. Provided the catalase is not present in excess the initial rapid reaction represents a small proportion of the total reaction possible, and as this condition obtained in most of the early investigations the results refer mainly to the subsequent steady rate. This is found to be directly proportional to the enzyme concentration, whereas the variation with peroxide concentration is more complicated. Above an optimum concentration as the peroxide is increased the reaction proceeds more slowly (Evans, 1907; Morgulis *et al.* 1926).

There is no doubt that the gradual decrease in the rate, after the initial rapid reaction is over, is due to enzyme destruction, and several kinetic equations have been developed to account for it in the papers of Yamasaki (1920), Morgulis (1921), Northrop (1924-5) and Williams (1927-8). There

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remain two problems. Enzyme destruction may be one factor determining the variation of the steady rate with peroxide concentration. Partial enzyme destruction may be responsible for the transition from the initial rapid rate to the slower steady rate—the transition from α - to β -activity which was examined in a previous paper (George, 1947).

Experiments have been carried out to establish the variation of the reaction rate with enzyme and peroxide concentration by measuring the rates in the early stages of the reaction for different initial concentrations. With this method it is possible to determine the true kinetics of the H_2O_2 decomposition unaffected by the slow destruction of the enzyme, which makes the kinetic analysis of H_2O_2 concentration changes during a prolonged experiment extremely difficult. In the second part of the paper experiments are described which show that enzyme destruction is not responsible for either the kinetics of the steady reaction or the transition from α - to β activity.

EXPERIMENTAL

Materials and methods

Enzyme preparations A purified preparation of liver catalase (CL1) was obtained from horse liver by the method described by Keilin & Hartree (1945). Erythrocyte catalase was prepared from 21 fresh defibrinated horse blood, the red corpuscles being washed and plasmolyzed, and the haemoglobin removed by the Tsuchihashi method as described by Keilin & Mann (1940) in their preparation of carbonic anhydrase. Catalase was extracted from the resulting clear yellowish fluid by the process used for liver catalase, i.e. adsorption on calcium triphosphate gel followed by elution and fractional precipitation with $(NH_4)_2SO_4$, this was done twice, NH_4OH being used for the first elution and Na_2HPO_4 for the second. Two specimens of erythrocyte catalase, CE1 and CE2 were obtained, CE1 was the purer sample, CE2 being extracted from the residues of the second adsorption. These specimens of catalase were analyzed for Fe, determined colorimetrically with 2,2'-dipyridyl, and for haemin, determined spectroscopically as pyridine haemochromogen (Keilin & Hartree, 1936), and compare favourably as regards purity with those used by Keilin & Hartree (1936) (Table 1). The low values for haemin are probably due to partial removal of the prosthetic group from the enzyme in the final stages of the

purification. Two other specimens of liver catalase, CL2 and CL3, kindly provided by Dr E. F. Hartree, have also been used. Dilute catalase solutions were made each day from the sterile stock solutions (see Table 1), and stored in ice and water in a thermos flask. Some dilutions were made in 0.02% gelatin which had no effect on the rate of the reaction, but reduced inactivation of the enzyme during storage.

A plasmolyzed red cell preparation used in later experiments was made by taking 1.0 ml of a red cell suspension from defibrinated horse blood, which had been washed twice with 0.9% NaCl and spun down, and diluting to 250 ml with distilled water.

Procedure The O_2 evolution was measured as previously described in Barcroft manometers at 20° and in the pressure gauge apparatus at 0° and 19.3–19.5° using the 'boat technique' of Meldrum & Roughton (1934) and George (1947). For the experiments in which dilute buffer solution was added to the reaction mixture during a run, flasks with a side tube of 1.5 ml capacity were used. In the experiments where H_2O_2 was added during a run, two dangling tubes were used, the first with a short platinum hook contained dilute catalase solution and the second with a long platinum hook contained the required amount of '100 vol' H_2O_2 . Control experiments with no catalase present showed the blank reaction to be negligible in the concentration range 0.1–0.5 M H_2O_2 , where '20 vol' H_2O_2 was used. For the range 1.0–5.0 M H_2O_2 , '100 vol' H_2O_2 was used, the contribution of the blank being determined at each concentration, and subtracted from the experimental value. Before use the '100 vol' H_2O_2 was brought to about pH 6.0 by adding a few drops of NaOH, bromocresol purple being used as indicator. In the majority of the experiments A.R. H_2O was employed, but in one series a freshly prepared sample was used.

In all the experiments the total volume of O_2 evolved was very small compared with the H_2O_2 concentration which can be assumed to remain constant throughout each experiment. Protocols for the two methods of measuring gas evolution in experiments in which the peroxide concentration was varied are given below. **Barcroft manometers** left-hand flask, 3.30 ml H_2O , right-hand flask, 0.30 ml 0.125 M phosphate buffer, pH 5.85, 0.10 ml 2% gelatin solution, 0.10 ml dilute catalase solution in dangling tube, H_2O and H_2O_2 to 3.30 ml to give required molarity. **Boats** Side 'a' 1.0 ml H_2O , 0.5 ml dilute catalase solution, 0.5 ml 0.2 M buffer solution, side 'b' 2.0 ml H_2O and H_2O_2 to give required molarity.

Buffers To determine the effect of different buffer solutions of the same pH, Sorensen's citrate buffer and Clark & Lubs's phthalate buffer were used as well as Sorensen's phosphate buffer. The solutions were prepared as described by Clark (1925), the pH values, determined electrometrically, were 5.86, 5.84 and 5.85 at 20° respectively, the apparatus having been standardized with 0.05 M potassium hydrogen phthalate solution, pH = 3.97 at 20°.

RESULTS

Variation of the oxygen evolution rate with enzyme concentrations

Barcroft manometers and the catalase specimen CL2 were used at 20°, the peroxide concentration being kept at a constant value and the enzyme con-

Table 1. Analysis of catalase specimens

(CL1 liver catalase, CE1 and CE2 erythrocyte catalase)

Analyses	Specimens		
	CL1	CE1	CE2
Dry weight (mg/ml)	34.1	5.2	5.4
Haemin (% on dry wt.)	0.51	0.47	0.50
Total iron (% on dry wt.)	0.14	0.11	0.09
Haemin iron (as % of total iron)	31	41	48

Keilin & Hartree (1936) found for three specimens 0.52, 0.55 and 0.37% haemin by weight.

centration varied For 0.01, 0.03 and 0.5 M- H_2O_2 a dilute catalase solution was employed and between 0.02 and 0.30 ml placed in the dangling tube, for

4.8 M- H_2O_2 a solution 10 times as concentrated was used The results are given in Table 2, and show that for each peroxide concentration the rate is directly proportional to the catalase concentration

Table 2 Variation of the oxygen evolution rate with enzyme concentration

(Barcroft manometers at 20° , catalase CL2 and 0.3 ml 0.125M phosphate buffer (pH 5.85) present in each experiment 10 Enzyme units (e u) equivalent to 1.84×10^{-4} mg CL2)

H_2O_2 (M)	E u	Rate at times after start ($\mu\text{l O}_2/\text{min}$)	Rate/e u
		0-1 min	
0.01	5	20	4.0
	10	42	4.2
	15	58	3.9
	20	73	3.7
	25	90	3.6
	30	111	3.7
0.03		2-3 min	
	2	14	7.0
	4	31	7.7
	6	43	7.2
	8	56	7.0
0.5		2-3 min	
	5	21	4.3
	10	42	4.2
	15	64	4.3
	20	32	4.1
4.8		2-3 min	
	10	7.5	0.75
	30	19.0	0.63
	50	29	0.58
	70	42	0.60
	90	58	0.64

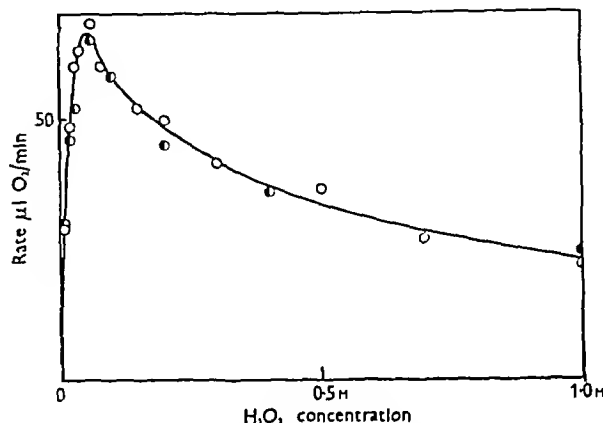


Fig 1 Variation of the O_2 evolution rate, between the second and third minute from the start of the reaction, with the peroxide concentration Measured with Barcroft differential manometers at 20° in the presence of 0.3 ml 0.125M phosphate buffer, pH 5.85, O—O, with liver catalase specimen CL1, 1.84×10^{-4} mg, ●—●, with erythrocyte catalase specimen CE1, 1.66×10^{-4} mg

Variation of the oxygen-evolution rate by liver or erythrocyte catalase with peroxide concentration

Barcroft manometers were used at 20° , the enzyme concentration being kept constant and the rate of O_2 evolution with different initial concentrations of peroxide determined The O_2 evolved was measured for 10 min at 0.5 and 1 min intervals, and from the figures obtained the rate over any given interval could be calculated Fig 1 shows the rates for liver

Table 3 Variation of the oxygen-evolution rate with peroxide concentration

(Barcroft differential manometers at 20° , 1.84×10^{-4} mg catalase CL2 and 0.3 ml 0.125M phosphate buffer (pH 5.85) present in each experiment)

H_2O_2 (M)	Rate at different times after beginning of reaction ($\mu\text{l O}_2/\text{min}$)				
	0-0.5 min	0.5-1 min	1-2 min	2-3 min	4-5 min
0.01	38	44	38	30	21
0.02	62	96	68	51	37
0.03	108	134	81	68	48
0.04	124	144	94	71	50
0.05	174	166	108	80	61
0.06	186	178	113	83*	63
0.07	214	222	119*	83*	65*
0.08	244	262*	119*	82	64
0.10	234	188	98	73	61
0.15	210	166	84	68	58
0.20	252	130	70	59	51
0.30	264	102	59	52	47
0.50	274*	71	47	42	37
0.70	226	56	35	33	30
1.00	180	62	34	29	27
2.00	100	25	15.5	13.5	13.5
3.40	66	21	9.5	8.5	7.5
4.80	54	14	7.5	7.5	7.5

* Indicates maximum rate

catalase CL1 and erythrocyte catalase CL1 over the range 0–1 M-H₂O₂, between the second and third minute from the start of the reaction, at which time most of the initial ‘burst’ is over (George, 1947) There is a very marked maximum in the rate at about 0.06 M H₂O₂, and the catalase specimens from the two different sources give identical curves. The inhibition of the reaction at high H₂O₂ concentrations is very marked, for with 1.0, 2.0 and 4.0 M-H₂O₂ the rate is 32, 21 and 8% respectively of the maximum rate at 0.06 M-H₂O₂.

No attempt has been made to compare the activities of the enzyme preparations on the basis of their haemin content. Catalase is an extremely active enzyme and the presence of about 1 × 10⁻⁶ g of inactive protein, and 1 × 10⁻⁸ g haemin or haemin degradation products is very unlikely to affect the rate of the reaction. For the kinetic analysis it is sufficient to show that with identical rates for the two specimens at, for instance, 1.0 M-H₂O₂, the same variation with H₂O₂ concentration is shown by each.

Similar results with liver catalase CL2 at 20° are shown in Table 3 which shows the initial rapid evolution of O₂ followed by a lower steady rate which was discussed in a previous paper (George, 1947). The maximum initial rate, as given by the O₂ evolved during the first half minute of the reaction, occurs at a high peroxide concentration between 0.3 and 0.7 M H₂O₂, whereas the maximum steady rate, measured by the O₂ evolved between the fourth and fifth minute from the start of the reaction, occurs at a much lower peroxide concentration—about 0.06–0.08 M-H₂O₂. The initial rates will be examined more fully in a later paper.

Variation of the oxygen-evolution rate with peroxide or enzyme concentration using plasmolyzed cells

To investigate the effect of impurities on the kinetics of the reaction, comparative experiments were carried out in the pressure gauge apparatus at 0° with erythrocyte catalase CE2 (1.73 × 10⁻³ mg) and a suspension of plasmolyzed red blood cells (1/250 dilution, 0.2 ml).

Fig. 2 shows that the same type of curve is given by the crude and the purified enzyme, and that the maximum occurs at the same H₂O₂ concentration. Table 4 shows that the rate is directly proportional to the concentration of lyzed cells. Since identical results are obtained with the purified enzyme extract and plasmolyzed red blood cells, the kinetics of the enzyme reaction are not affected by chance impurities in the enzyme preparation.

Effect of different samples of hydrogen peroxide on the oxygen evolution rate

The inhibition of the reaction observed at high peroxide concentrations might be due to an inhibitory substance present in the specimen of H₂O₂.

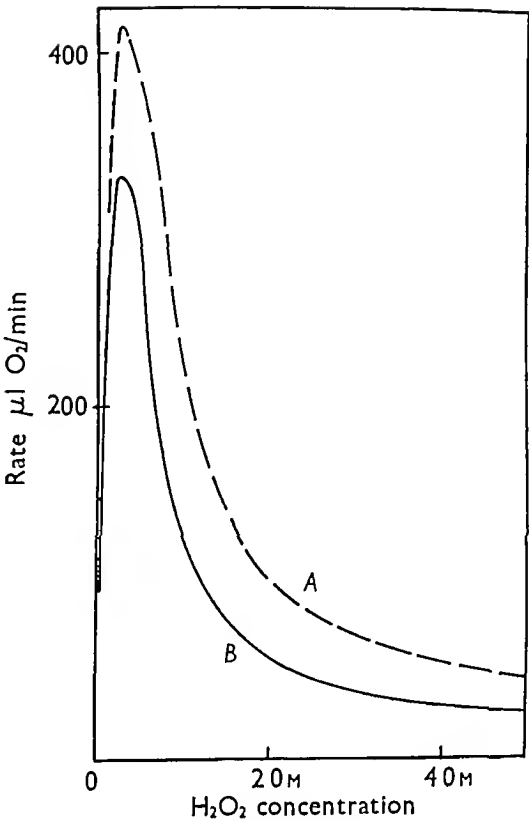


Fig. 2 A comparison of the variation of the O₂ evolution rate with peroxide concentration for a dilute suspension of lyzed red blood cells (curve A) and the purified erythrocyte catalase specimen CE2 (curve B). Measurements of the rate in μl O₂/min made between 10 and 40 sec from the start of the reaction with the pressure gauge apparatus at 0°, 0.5 ml 0.2M phosphate buffer (pH 5.85), 1.73 × 10⁻³ mg catalase (CE2) or 0.2 ml 1/250 dilution lyzed cells present.

Table 4 Variation of the oxygen evolution rate with the concentration of plasmolyzed red blood cells

(Pressure gauge apparatus at 0° in the presence of 0.5 ml 0.2M phosphate buffer, pH 5.85. Lyzed cell concentration (L.C.) in ml 1/250 dilution. Rate measured between 10 and 40 sec from the start of the reaction.)

H ₂ O ₂ (M)	L.C. (ml)	Rate (μl O ₂ /min)	Rate/ml enzyme
0.2	0.05	90	1800
	0.10	180	1800
	0.15	295	1980
	0.20	400	2000
1.0	0.1	85	850
	0.2	200	1000
	0.3	305	1020
	0.4	405	1010
5.0	0.2	40	200
	0.4	120	300
	0.6	180	300
	0.8	210	262

used. A sample of H_2O_2 was, therefore, prepared in the laboratory from Na_2O_2 and H_2SO_4 and purified by vacuum distillation (Kilpatrick, Reiff & Rice, 1926). Neither the standard sample nor the fresh sample showed any fluorescence when illuminated with strong ultraviolet light. Liver catalase CL2 was used for the determinations which were carried out in Barcroft manometers at 20° . Table 5 shows

Table 5 *Comparison of the oxygen evolved from the standard peroxide and freshly prepared peroxide*

(Barcroft manometers at 20° , 1.84×10^{-4} mg catalase CL2 and 0.3 ml. 0.125 M phosphate buffer (pH 5.85) present in each experiment. The average of five separate determinations given for each sample.)

H_2O_2 (M)	Time after start (min)	O_2 evolved ($\mu\text{l}/\text{min}$)	
		Standard H_2O_2	Fresh H_2O_2
0.5	1-2	30.8	31.5
	2-3	29.8	29.6
	4-5	24.5	24.0
0.07	1-2	73.7	73.0
	2-3	52.5	51.4
	4-5	40.3	39.6

that there is a precise correspondence between the results for both specimens of H_2O_2 , so the inhibition of the reaction at high H_2O_2 concentrations is not due to any inhibitory substance present in the peroxide.

Effect of different buffer solutions of the same pH on the oxygen evolution rate by catalase

Anions are known to inhibit the decomposition of H_2O_2 by catalase owing to competition for the ferric iron of the haem in between OH^- and the other

anions (Agner & Theorell, 1946), which is more marked the greater the H^+ ion concentration. It was desirable to find out whether the inhibition observed at high H_2O_2 concentrations arises from a complex reaction involving the constituents of the buffer solution. Experiments were made using the pressure-gauge apparatus at 19.3 – 19.5° with liver catalase CL3 to compare the effect of phosphate, citrate and phthalate buffers of pH values 5.85, 5.86 and 5.84 respectively, at concentrations between 0.005 and 0.1 M. The results are given in Table 6 for two concentrations of substrate (0.1 and 1.0 M- H_2O_2). The results show clearly that at pH 5.84–5.86 the rates are the same at 1.0 M- H_2O_2 and independent of the nature and concentration of the buffer solution used. At 0.1 M H_2O_2 there may be a slight inhibition with the phthalate buffer, but the variation in the figures is of the same order as the experimental error. With all these buffers the steady rate with 0.1 M- H_2O_2 is between 2.0 and 2.4 times as great as the steady rate with 1.0 M- H_2O_2 as observed in the previous experiments, and there is no systematic fall in the rate as the buffer concentration is increased. This shows clearly that reactions of the anion or cation of the buffer solution do not play any part in determining the kinetics of the peroxide decomposition, and are not responsible for the inhibition of the decomposition observed at high H_2O_2 concentrations.

Reversibility of the inhibition of catalase by high peroxide concentrations

The question whether destruction of enzyme accounts for the reaction kinetics can be settled by experiments in which water or peroxide is added

Table 6 *Comparison of the oxygen evolution rates with different buffers of the same pH*

(Pressure gauge apparatus at 19.3 – 19.5° , catalase CL3, 7.3×10^{-4} mg.)

Buffer			Rates ($\mu\text{l O}_2/\text{min}$) at time after start of reaction			
Type	pH	Vol (0.2 M solution)	0.1 M H_2O_2		1.0 M H_2O_2	
			10-60 sec	2-4 min	10-60 sec	2-4 min
Phosphate	5.85	0.1	950	248	199	109
		0.5	965	240	199	106
		1.0	995	248	208	113
		1.5	990	252	211	117
		2.0	1040	235	—	—
		Av	988	245	204	111
Citrate	5.86	0.1	1060	232	204	113
		0.5	988	226	198	109
		1.0	—	—	—	—
		1.5	—	—	228	114
		2.0	960	218	—	—
		Av	996	225	210	112
Phthalate	5.84	0.1	955	223	202	102
		0.5	955	218	199	100
		2.0	950	215	230	103
		Av	953	219	210	102

during the reaction, for if complete reversibility is observed when the substrate concentration is brought to the optimum, then it is certain that enzyme destruction plays no part in determining the kinetics.

In the dilution experiments a buffer solution was added rather than water alone so that the ionic strength did not change, as an added safeguard that the observed effect is due only to the reaction between catalase and H_2O_2 . The experiments were made in Barcroft manometers at 20° , a total of 1.5 ml being used in each flask the O_2 evolution was followed for 4.5 or 5 min, each experiment being done in triplicate. Catalase CL3 was employed, 0.1 ml (1.9×10^{-5} mg) being placed in the dangling tube the concentration after mixing was about one fifth of that used in the previous experiments. With the usual amount of catalase the amount of O_2 evolved during the 5 min of this experiment might amount to one quarter of the total H_2O_2 present, giving a substrate concentration below the optimum which would tend to mask the increase in the rate which is being looked for. Measurements of O_2 evolution were carried out at 0.01, 0.06, 0.36 and 1.46 M- H_2O_2 , the appropriate blanks for the H_2O_2 alone being subtracted from the observed values. Two sets of experiments were made in which dilute buffer solution was added during the reaction, the flask with a side arm of capacity about 1.5 ml being used. Typical protocols are given.

(a) *Concentration change from 0.6 to 0.06 M- H_2O_2*
Left hand flask: 1.5 ml H_2O . Right-hand flask: 0.094 ml 1.92 N H_2O_2 , 0.06 ml 0.2 M-phosphate buffer (pH 5.85), 0.10 ml dilute catalase solution in dangling tube, total = 0.25 ml 0.36 M- H_2O_2 , side arm, 0.95 ml H_2O , 0.30 ml 0.2 M-phosphate buffer (pH 5.85), total 1.25 ml, after mixing total volume = 1.50 ml 0.06 M H_2O_2 . With this arrangement the evolution of O_2 can first be determined for 0.25 ml of 0.36 M H_2O_2 , and then, by allowing the dilute buffer solution to run into the flask by rotating the side arm, measurements can be continued for 1.5 ml of 0.06 M- H_2O_2 , any alteration in the rate is to be attributed only to the change in H_2O_2 concentration. This was checked by an experiment where the H_2O_2 concentration was kept constant (Fig. 3, curve A), the O_2 evolution from 0.25 ml of 0.36 M H_2O_2 with 0.10 ml dilute catalase being the same as that from 1.50 ml of 0.36 M- H_2O_2 with 0.10 ml dilute catalase, i.e. the same amount but one sixth the previous concentration. Curve B gives the O_2 evolution for 1.50 ml of 0.06 M- H_2O_2 with 0.10 ml dilute catalase. In experiments illustrated by curves C and D the reaction was started with 0.25 ml of 0.36 M- H_2O_2 and after 2 and 1 min respectively 1.25 ml of dilute buffer solution were added according to the protocol above, in both

cases the O_2 evolution after dilution increases and follows the points on curve B, the appropriate curve for the lower H_2O_2 concentration. The inhibition observed with 0.36 M- H_2O_2 can, therefore, be reversed by dilution during the reaction, the reversibility being quantitative to within experimental error. The rates of O_2 evolution, $\mu\text{l O}_2/\text{min}$, measured over the period 2–4.5 min from curves A, B and C are 7.0, 10.5 and 10.4 respectively. Curve D,

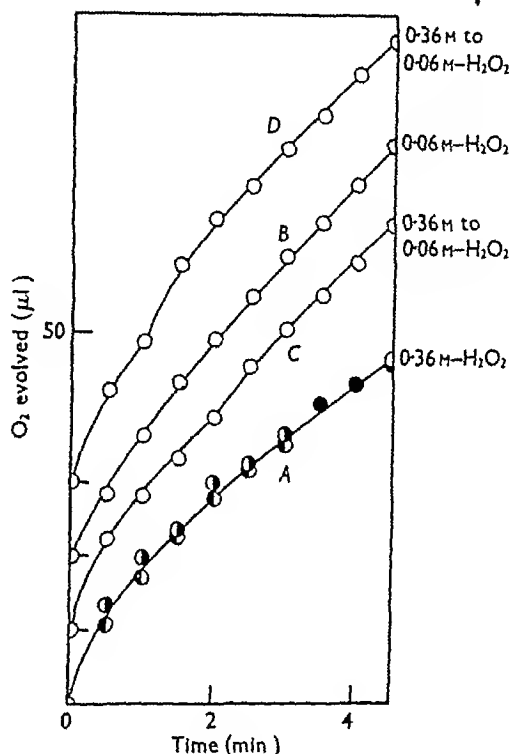


Fig. 3. O_2 evolution curves for 0.36 and 0.06 M H_2O_2 with 1.9×10^{-5} mg of catalase (CL3) in each experiment. Curve A: 0.36 M H_2O_2 , 0.25 ml solution points \bigcirc , 1.50 ml solution points \bullet . Curve B: 0.06 M H_2O_2 , 1.50 ml solution. Curve C: from 0 to 2 min 0.25 ml 0.36 M H_2O_2 , then 1.25 ml dilute buffer solution added from a side arm giving 1.50 ml 0.06 M H_2O_2 . Curve D: as for curve C but addition of dilute buffer solution at the end of the first minute. Measurements made with Barcroft differential manometers at 20° in phosphate buffer (pH 5.85), concentration in each experiment 0.024 M.

illustrating the effect of dilution at the end of the first minute of the reaction, will be discussed later when the possibility of enzyme destruction as the cause of the decay of the initial rapid reaction is examined.

(b) *Concentration change from 0.06 to 0.01 M- H_2O_2*
The results in Table 3 show too that dilution from 0.06 to 0.01 M- H_2O_2 should cause a marked decrease in the rate. This was tested in a similar way to the foregoing experiments, using 0.32 N H_2O_2 in place of 1.92 N- H_2O_2 . The average results for these separate

determinations of O_2 evolution with each H_2O_2 concentration are given in Fig 4. Curves *A* and *B* show the O_2 evolution for 1.50 ml of 0.01 M- H_2O_2 and 0.25 ml of 0.06 M H_2O_2 , the rate being greater at the higher H_2O_2 concentration, curve *C* gives the O_2 evolution for the reaction starting with 0.25 ml of 0.06 M- H_2O_2 and adding 1.25 ml of buffer solution after 2 min, and shows the expected decrease in the rate. The rates of O_2 evolution, measured over the period 2–4.5 min from curves *B*, *A* and *C* are 10.4, 4.8 and 4.4 $\mu l O_2/\text{min}$ respectively.

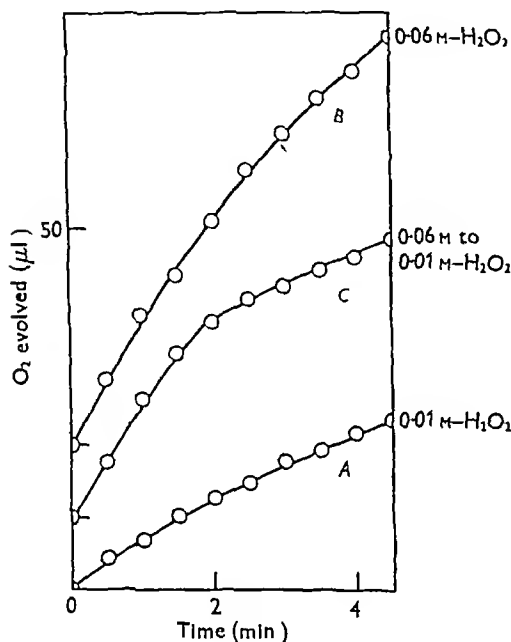


Fig 4 O_2 evolution curves for 0.01 and 0.06 M H_2O_2 with 1.9×10^{-5} mg catalase (CL3) in each experiment. Curve *A* 0.01 M H_2O_2 , 1.50 ml solution. Curve *B* 0.06 M H_2O_2 , 0.25 ml solution. Curve *C* from 0 to 2 min 0.25 ml 0.06 M H_2O_2 , then 1.25 ml dilute buffer solution added from a side arm giving 1.50 ml 0.01 M H_2O_2 . Measurements made with Barcroft differential manometers at 20° in phosphate buffer (pH 5.85), concentration in each experiment 0.024 M.

(c) *Concentration change from 0.01 to 0.36 M- H_2O_2*
Two sets of experiments were then made in which a small amount of '100 vol' H_2O_2 was added during the reaction from a second dangling tube with a long platinum hook. The dilute catalase solution was contained in the first dangling tube, which had a short platinum hook, and by careful tapping could be dislodged without upsetting the second tube holding the concentrated H_2O_2 . Protocol: left-hand flask, 1.5 ml H_2O , right hand flask, 0.094 ml 0.32 N- H_2O_2 , 0.40 ml 0.2 M-phosphate buffer (pH 5.85), 0.90 ml H_2O , 0.10 ml catalase (= 1.50 ml 0.01 M- H_2O_2), 0.05 ml 10.5 M- H_2O_2 in second dangling tube. Total volume = 1.50 ml 0.36 M- H_2O_2 . It is important to show that by increasing the H_2O_2

concentration the rate can also be increased, for this would confirm that the inhibition noted at high H_2O_2 concentrations is not caused by an irreversible oxidative degradation of the enzyme. This can be tested in the concentration range 0.01–0.36 M- H_2O_2 . The direct determination of the rates at these two concentrations recorded in Table 3 shows about a twofold increase. Fig 5 shows the average of three separate determinations of O_2 evolution at each of these H_2O_2 concentrations, curve *A*, 1.50 ml of 0.01 M H_2O_2 , and curve *B*, 1.50 ml of 0.36 M- H_2O_2 . Curve *C* gives the O_2 evolution for the reaction

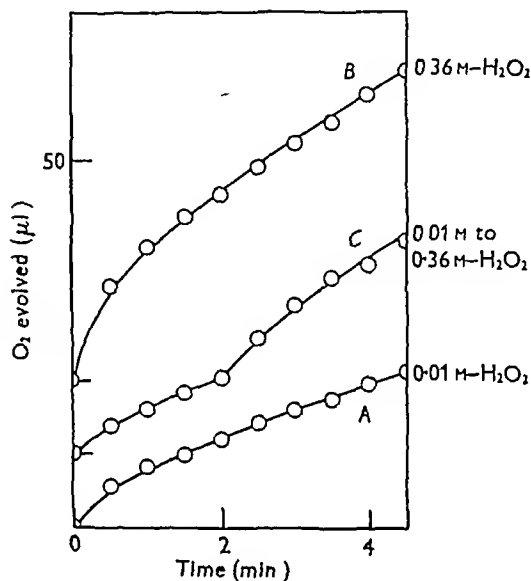


Fig 5 O_2 evolution curves for 0.01 and 0.36 M H_2O_2 with 1.9×10^{-5} mg catalase (CL3) in each experiment. Curve *A* 0.01 M H_2O_2 , 1.50 ml solution. Curve *B* 0.36 M H_2O_2 , 1.50 ml solution. Curve *C* from 0 to 2 min 1.50 ml 0.01 M- H_2O_2 , then 0.05 ml 10.5 M H_2O_2 added from a second dangling tube giving 1.55 ml 0.36 M H_2O_2 . Measurements made with Barcroft differential manometers at 20° in phosphate buffer (pH 5.85), concentration in each experiment 0.024 M.

starting with 1.50 ml of 0.01 M H_2O_2 and adding 0.05 ml of 10.5 M- H_2O_2 from the second dangling tube after 2 min to give 0.36 M H_2O_2 . A marked increase in the rate is observed, which corresponds to that expected for the increase in H_2O_2 concentration. The rates of O_2 evolution measured over the period 2–4.5 min, from curves *A*, *B* and *C*, are 3.7, 7.0 and 7.4 $\mu l O_2/\text{min}$ respectively.

(d) *Concentration change from 0.06 to 1.46 M- H_2O_2*
The inhibitory effect of strong H_2O_2 which was observed in the direct determinations of the rate at different H_2O_2 concentrations (Fig 2 and Table 3) was confirmed by adding peroxide during the reaction to bring the concentration from 0.06 to 1.46 M H_2O_2 when the rate should fall to about one third of its previous value. In Fig 6, curves *A* and *B* refer to the O_2 evolution from 1.50 ml of 1.46 and

0.06 M- H_2O_2 respectively, while curve *C* gives the O_2 evolved from 1.30 ml of 0.06 M- H_2O_2 for 2 min, when 0.20 ml of 10.5 M- H_2O_2 was added and readings taken for a further 3 min. The rate decreases quantitatively corresponding to the

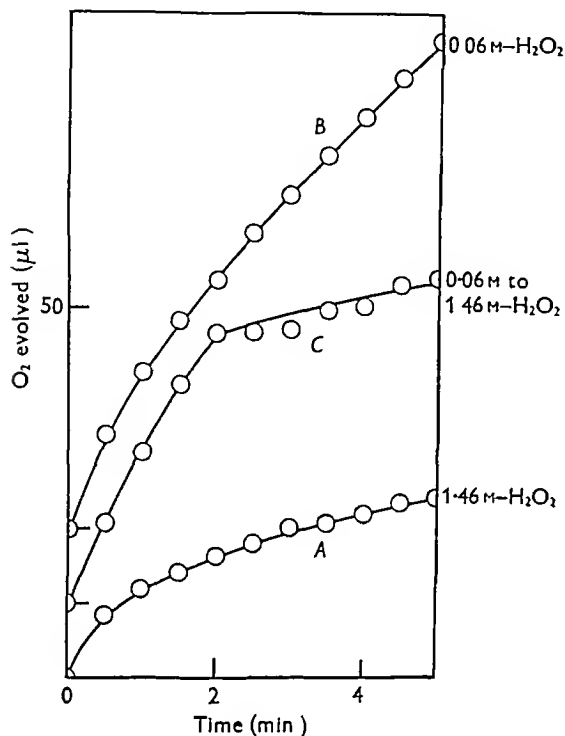


Fig 6 O_2 evolution curves for 1.46 and 0.06M H_2O_2 with 1.9×10^{-5} mg catalase (CL3) in each experiment. Curve *A* 1.46M- H_2O_2 , 1.50 ml solution. Curve *B* 0.06M- H_2O_2 , 1.50 ml solution. Curve *C* from 0 to 2 min 1.30 ml 0.06M H_2O_2 , then 0.20 ml 10.5M- H_2O_2 added from a second dangling tube giving 1.50 ml 1.46M- H_2O_2 . Measurements made with Baroroft differential manometers at 20° in phosphate buffer (pH 5.85), concentration in each experiment 0.024M.

change in H_2O_2 concentration. The rates of O_2 evolution, measured over the period 2–4.5 min, from curves *B*, *A* and *C* are 10.9, 2.9 and 2.9 $\mu\text{l O}_2/\text{min}$ respectively.

DISCUSSION

The experiments described above show that the rate of decomposition of H_2O_2 by catalase is directly proportional to the enzyme concentration and shows a complex variation with the H_2O_2 concentration. Below 0.06 M- H_2O_2 the rate is directly proportional, between 0.06 and 0.08 M- H_2O_2 there is a maximum in the rate, above 0.08 M- H_2O_2 further increase in the peroxide decreases the rate. These results, particularly the inhibition at high H_2O_2 concentration, are in no way determined by the purity of the enzyme, the presence of inhibitors in the

peroxide or the nature and concentration of the buffer solution used. The quantitative reversal of the inhibition shown by the addition of dilute buffer solution or peroxide during the reaction shows that the phenomenon is not due to destruction of the enzyme. The remaining problem to be settled is whether or not partial enzyme destruction is responsible for the transition from the initial rapid rate to the steady rate. The experiment recorded in Fig 3, curve *C*, shows that it is not

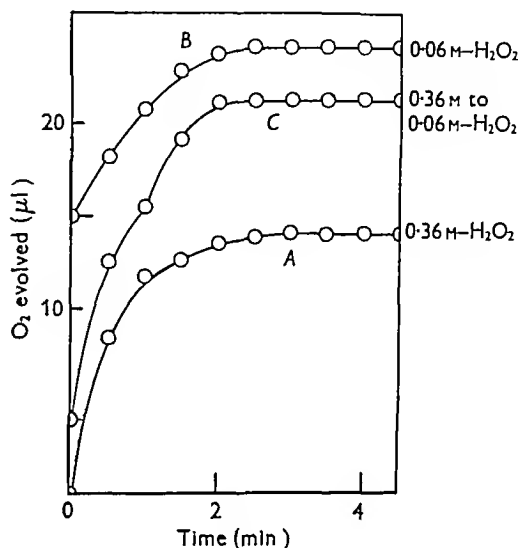


Fig 7 O_2 evolved during the initial rapid reaction with 0.36 and 0.06M H_2O_2 , calculated from the O_2 evolution curves in Fig 3 by subtracting the O_2 evolved in the steady reaction. Curve *A* (from Fig 3*A*) 0.36M H_2O_2 , 0.25 ml solution. Curve *B* (from Fig 3*B*) 0.06M H_2O_2 , 1.50 ml solution. Curve *C* (from Fig 3*D*) from 0 to 1 min 0.25 ml 0.36M H_2O_2 , then 1.25 ml dilute buffer solution added from a side arm giving 1.50 ml 0.06M H_2O_2 .

The experiment is one of the series in which dilute buffer solution was added during the reaction, the addition being made at the end of the first minute, i.e. during the period of the rapid reaction. The concentration change was from 0.36 to 0.06 M H_2O_2 . The effect of this dilution during the initial rapid reaction can best be seen by subtracting from all the experimental points in Fig 3 the appropriate contribution of the steady rate. This is done in Fig 7, where curves *A*, *B* and *C* give the O_2 evolution for the calculated rapid reaction for 0.36, 0.06 and 0.36 \rightarrow 0.06 M- H_2O_2 , from the experimental points in Fig 3, curves *A*, *B* and *D*. The rapid reaction is over more quickly the higher the peroxide concentration. With 0.36 M- H_2O_2 , 83% is over in the first minute, with 0.06 M- H_2O_2 , 65%. Curve *C*, Fig 7, shows that after dilution a greater quantity of O_2 is evolved in completing the initial rapid reaction than is evolved in the more concentrated solution. This suggests

that the transition to the steady rate cannot be due to any partial destruction of the enzyme. If it were, no increase in O_2 evolution would be observed on dilution. The explanation for this transition from α - to β activity in the decomposition of H_2O_2 by catalase should be sought in the series of unit reactions involved in the peroxide decomposition. In this respect there is a similarity in the kinetics of the enzymatic decomposition and of the reaction catalyzed by ferrous iron (George, 1947), even though the iron in catalase is in the ferric state.

SUMMARY

1 The evolution of oxygen from hydrogen peroxide at pH 5.58 by liver catalase, erythrocyte catalase and a suspension of plasmolyzed red blood corpuscles has been measured manometrically, and by the 'boat technique' of Meldrum & Roughton (1934) using a pressure gauge for following rapid rates. There is an initial rapid evolution of O_2 followed by a slower steady evolution—a transition from α - to β activity.

2 Identical kinetic relationships are obtained with the three specimens of catalase. The rate is directly proportional to the catalase concentration. The variation of the rate with peroxide concentration shows a maximum at about 0.07 M- H_2O_2 ; further increase in the H_2O_2 concentration decreasing the rate very markedly.

3 These kinetic relationships, particularly the inhibition at high H_2O_2 concentrations, are in no way determined by the purity of the enzyme, the presence of inhibitors in the peroxide or the nature and concentration of the buffer solution used.

4 The quantitative reversal of the inhibition shown by addition of dilute buffer solution or peroxide during the reaction shows that enzyme destruction plays no part in determining these kinetic relationships.

5 Dilution during the period of initial rapid activity shows a similar reversibility which suggests that the transition from α - to β activity is not caused by the partial destruction of the enzyme.

I wish to thank Prof. D. Keilin, F.R.S., for his stimulating interest and kind encouragement in this work.

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Activity of the Succinic Dehydrogenase-cytochrome System in Different Tissue Preparations

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The object of this paper is the study of the effect of different factors on the rate of aerobic oxidation of succinic acid by various tissue preparations. From the results obtained it is apparent that there are broadly speaking two types of factor: first, those which may react directly and specifically with one or more components of the succinic dehydrogenase-cytochrome system or of other catalytic mechanisms linked to this system, and secondly those which, by modifying the colloidal state of the enzyme prepara-

tions, may markedly increase or decrease their activity. The significance of the latter type of factor has not hitherto been fully recognized, with the result that the mode of action of a number of inhibitors and activators on the succinic system in tissue extracts has been misinterpreted, and the activators have erroneously been considered as essential links in the catalytic chain which brings about the aerobic oxidation of succinate. This study will enable us to examine critically the claims of

previous workers to have discovered or even isolated such additional catalytic intermediates in the succinic system

MATERIALS AND METHODS

The preparation of heart muscle (from horse unless otherwise stated) and pigeon breast muscle extracts and of de-natured globin (from recrystallized horse oxyhaemoglobin) have already been described (Keilin & Hartree, 1947*a, b*). If prepared exactly according to our description the heart preparation will yield, on 20 fold dilution with 0.1M-phosphate (pH 7.3), a homogeneous opalescent fluid which shows no sign of aggregation on standing for several hours at room temperature. The appearance of a flocculent precipitate indicates either that the pH became too low (<5.5) during the isolation of the enzyme material, or that it was not kept cold at that stage. Although such materials may show catalytic activity, they are unsuitable for the experiments described in this paper. Cytochrome *c* was prepared from horse heart (Keilin & Hartree, 1945) and a 1% solution of the pigment, containing 0.34% Fe, was generally used. In certain experiments cytochrome *c* containing 0.43% Fe was used.

Kidney oxidase preparations The cortical layer of 1 horse kidney was passed through a Latapie mincer, stirred 10 min with 1 l distilled water and pressed out in muslin. The pulp was stirred with a further 1 l water and pressed out again. The combined fluids were centrifuged to remove solid particles and the cloudy fluid cooled to 0° was brought to pH 5.5 with N acetic acid. The precipitate was immediately centrifuged down, washed by centrifuging with 0.02M KH_2PO_4 , and the solid material resuspended in its own volume of 0.1M phosphate buffer (pH 7.3).

Vacuum dried preparations Heart-muscle preparation (20 ml) in a 500 ml beaker was evacuated to 0.1 mm Hg in a desiccator over KOH and concentrated H_2SO_4 . Within 10–20 min the material froze as a result of the rapid evaporation. The desiccator was then detached from the pump and allowed to stand evacuated overnight. In this way a light flaky product was obtained, very similar in appearance to a freeze dried protein.

Frozen oxidase preparations Heart muscle preparations were frozen three times, either in liquid air or in an ice salt freezing mixture, with complete thawing after each freezing. This treatment resulted in an irreversible flocculation of the colloidal suspension.

Glyoxaline buffer As veronal inhibits succinic dehydrogenase, phosphate free buffers (pH 7.3) were made up from 4(5) methylglyoxaline (Windaus & Knoop, 1905) according to the description of Kirby & Neuberger (1938).

The oxidation of succinate was followed in differential manometers at 39° with 3.3 ml fluid in each flask as already described (Keilin & Hartree, 1947*b*). Unless otherwise stated, the concentration of buffer was 0.07M with respect to phosphate or glyoxaline. The right hand flasks received the appropriate oxidase preparation and 0.2 ml 1% cytochrome *c*. Sodium succinate was added from a dangling tube, its concentration after mixing with the contents of the flask being 0.04M. All experiments were carried out at pH 7.3. When frozen or dried preparations were tested this fact is stated, in all other cases the fresh material was used. The term Q_{O_2} used in this paper corresponds to $\mu\text{l O}_2$ taken up/mg dry wt of preparation/hr.

EXPERIMENTAL

A Effect of varying concentrations of phosphate and glyoxaline buffers

Our experiments have shown that phosphate buffer cannot be considered as an indifferent medium for the study of the succinic dehydrogenase cytochrome system.

Fig 1, which summarizes the experimental results obtained with the heart muscle preparation before and after vacuum drying, clearly indicates that the rate of oxidation of succinic acid increases with the decrease in phosphate concentration down to 0.15M, but at lower concentrations the rate falls again. In the case of the undried material these changes in the activity of the preparation follow the

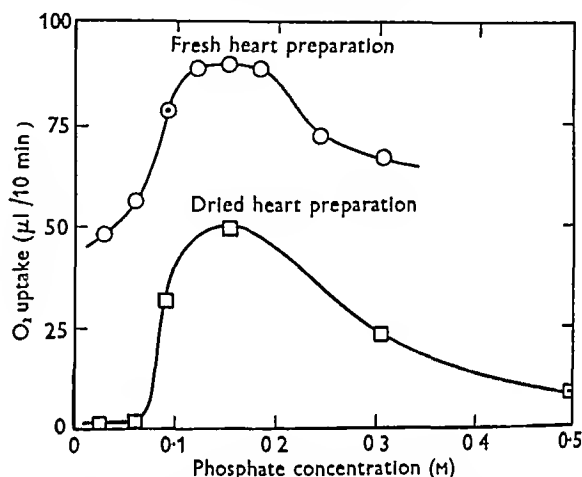


Fig 1 Effect of phosphate buffer concentration on the rate of O_2 uptake of fresh and vacuum dried heart preparations in presence of succinate and cytochrome *c*. Dry wt of preparation $\approx 1.1 \text{ mg}$, temp, 39°, pH, 7.3

degree of dispersion of the protein, the most complete dispersion being at 0.15M phosphate with a tendency towards flocculation as the concentration of phosphate is either decreased or increased. When phosphate is replaced by the glyoxaline buffer the rate of oxidation of succinate increases with the decrease of buffer concentration. For such experiments glyoxaline buffer must be used instead of phosphate in the preparation of the heart-muscle extract. The peculiar effect of phosphate can be illustrated from experiments where the total salt concentration (phosphate + chloride) remained constant while the phosphate concentration, denoted as [phosph], was varied. In such a case there is a definite activation by phosphate when comparison is made with control experiments in glyoxaline buffer (Table 1). * The combination of this activation by [phosph] and the general salt effect presumably gives rise to the maxima in Fig 1. No optimal effect of [phosph] is shown by kidney preparations where the reaction rate falls off with increasing

* Throughout this paper we use the term 'activation' to indicate an increase in the rate of O_2 uptake of the entire catalytic system.

Table 1 *Comparison of the effects of phosphate and glyoxaline buffers on the oxidation of succinate by a heart oxidase preparation*

(Total volume of oxidase (0.05 ml) + cytochrome c + succinate = 1.0 ml NaCl and buffers 0.1 M, temperature, 39°, pH, 7.3)

	O ₂ uptake (μ l/20 min)	
	In phosphate	In glyoxaline
Oxid + cyt c + succ + 1.3 ml buffer + 1.0 ml NaCl	87	97
Oxid + cyt c + succ + 1.8 ml buffer + 0.5 ml NaCl	116	100
Oxid + cyt c + succ + 2.3 ml buffer + 0 ml NaCl	138	99

Table 2 *The effect of phosphate concentration on the oxidation of succinate by a kidney preparation, and the effect of cytochrome c*

(Kidney oxidase (0.25 ml), succinate, temp, 39°, pH, 7.3)

Phosphate conc (M)	μ l O ₂ uptake in 10 min	
	No cyt c	With cyt c
0.015	20	96
0.03	14	77
0.15	8	18
0.30	1	9

[phosph] (Table 2) The rate of reduction of methylene blue in Thunberg vacuum tubes by succinate and the various preparations is independent of [phosph] over a wide range

B *Effect of adenosinetriphosphate (ATP)*

Biró & Szent Györgyi jun (1946) tested the effect of ATP on the oxidation of succinic acid by a preparation obtained from rabbit skeletal muscle minced and washed twice in 20 volumes of water. The maximum Q_{O_2} of this muscle preparation at 37° without and with ATP was found by them to be 2.5 and 5 respectively. They concluded that 'the activity of the succinoxidase is greatly dependent on the presence of ATP'.

As the activity of their enzyme system was very low we considered it interesting to determine the effect of ATP on our enzyme preparations, the Q_{O_2} of which varies between 500 and 700. The experiments, carried out in the usual way, showed that the Q_{O_2} of a horse heart preparation in phosphate buffer without and with 0.003 M ATP (5 mg/manometer flask) was 620 and 540 respectively. In other words, ATP produced about 13% inhibition. Experiments carried out on a heart-muscle extract prepared and tested in glyoxaline buffer gave similar results, namely, ATP, in presence or in absence of K ions, invariably produced about 15% inhibition. The glyoxaline heart muscle preparation was found to contain only a trace of inorganic phosphate and its adenosinetriphosphatase (ATPase) activity was very low. Thus 0.2 ml of this preparation (i.e. 4 times the

amount used in manometric experiments) contained 5 μ g inorganic P and when incubated with 5 mg ATP at 39° for 50 min, hydrolyzed only 8% of the substrate.

Since the heart muscle oxidase produced by our method contains very little ATPase further experiments were carried out on a pigeon breast muscle preparation (Keilin & Hartree, 1947b) using both phosphate and glyoxaline buffers for extraction. Several samples were collected in the course of fractionation of the muscle and these differed in their activities with respect to succinate, and in their contents of myosin and ATPase. An examination of these fractions in glyoxaline and in phosphate buffer showed that the addition of ATP produced inhibitions varying from 25 to 37%.

We can say in conclusion that the results of our experiments, unlike those of Biró & Szent-Györgyi jun (1946), show that ATP inhibits the catalytic activity of highly active preparations oxidizing succinic acid. The inhibitory effect of ATP is, in fact, of the same order as that produced by an equivalent concentration of inorganic pyrophosphate.

C *Effect of proteins on the activity of the succinic systems in different preparations*

The activation by denatured globin of a heart-muscle preparation oxidizing succinate was recorded in a previous paper (Keilin & Hartree, 1947b) under a single set of experimental conditions. Further work revealed that other proteins exhibited the same property, and that the magnitude of the effect was greatly dependent upon phosphate concentration. Such experiments showed that, except at very high [phosph], the activity of a heart preparation in presence of added proteins was independent of [phosph] and that the effect of protein in sub-optimal [phosph] was to raise the activity of the preparation to the optimal level (Table 3). From

Table 3 *The effect of phosphate concentration on the oxidation of succinate by a heart preparation ± added protein*

(Oxidase (0.04 ml), succinate and cytochrome c, 0.3 ml 1.4% denatured globin or 0.3 ml horse plasma, temp, 39°, pH, 7.3)

Phosphate conc (M)	O ₂ uptake (μ l/30 min)		
	Alone	+ Plasma	+ Globin
0.03	198	320	349
0.09	294	348	—
0.15	325	340	331
0.30	279	279	—

this it follows that the effect of proteins can only be studied at low [phosph] and the concentration chosen for subsequent work was 0.07 M. It is important to note that denatured globin is completely precipitated at the pH of our experiments (7.3). From the results of experiments carried out

with an ox-heart preparation and summarized in Table 4 it is evident that proteins generally, but not their digests, activate the oxidation of succinate, and that denatured and consequently insoluble proteins are the most efficient in this respect. Similar results were obtained with a horse heart preparation.

Table 4 *The effects of various substances on the aerobic oxidation of succinate by a pig-heart preparation*

(Phosphate (0.07M), oxidase (0.05 ml), succinate, cytochrome c, temp, 39°, pH, 7.3)

Substance added	Relative rate of O ₂ uptake
None	100
Crystalline serum albumin (2%)	219
Peptone (2%)	117
Gelatine (2%)	200
Casein (commercial (2%))	171
Tryptic digest of casein (2%)	122
Horse plasma (0.4 ml)	210
Methaemoglobin (2%)	206
Denatured globin (2%)	285*
Boiled methaemoglobin (2%)	244*
Ca ₃ (PO ₄) ₂ gel (30 mg)	199
CaCl ₂ + AlCl ₃ (each 4 × 10 ⁻⁴ M)†	165
Boiled oxidase (0.1 ml)	167
Vacuum-dried oxidase (4 mg)	180‡

* Precipitate of protein in manometer flask

† Concentrations used by Schneider & Potter (1943)

‡ Corrected for O₂ uptake of dried preparation

Pigeon-breast muscle preparations, on the other hand, are not affected by proteins (Keilin & Hartree, 1947b).

D *The effect of cytochrome c compared with that of indifferent proteins*

The increased rate of oxidation of succinate by heart-muscle preparations obtained on adding proteins is of the same order as that given by cytochrome c. This raises the question whether cytochrome c may act as an indifferent protein as well as a specific oxido-reduction catalyst. The study of this question is most conveniently carried out with certain modified types of preparations which will be described below.

(1) *Experiments with frozen heart muscle preparations*
The best results were given by a pig heart preparation preserved 2-3 days at 5° after freezing 3 times in an ice salt mixture. The rate of oxidation of succinate by this preparation was studied in presence of (a) horse blood serum, (b) cytochrome c (0.43% Fe), (c) alkali denatured cytochrome c, (d) denatured globin and (e) denatured globin treated with alkali in the same way as the cytochrome c. The alkali treatment consists in 18 hr contact with N NaOH followed by neutralization with HCl and 4 hr dialysis to remove excess of salt. We have previously shown (Keilin & Hartree, 1940) that such treatment renders cytochrome c catalytically inactive in an oxidizing system obtained from

tissue extracts. The results of these experiments are summarized in Fig. 2 where the activating effects of various proteins are plotted against log protein concentration. The order of efficiency is cytochrome c > denatured globin > serum proteins, but whereas alkali treatment reduces cytochrome c to the serum level, it does not affect denatured globin. These experiments distinguish clearly between the influence of added cytochrome c as an indifferent protein and its catalytic effect as an essential oxido-reduction catalyst. No explanation of the maxima in certain curves is forthcoming from our experiments.

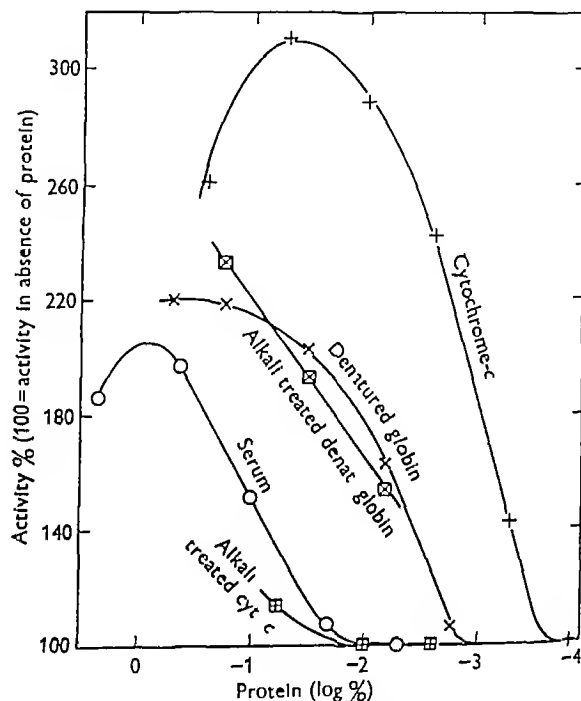


Fig. 2 Effect of cytochrome c, denatured globin and serum proteins on the oxidation of succinate by a frozen heart-muscle preparation (1.1 mg). Ordinates activity expressed as percentage of that in absence of the various proteins (36 μ l O₂/10 min). Abscissae log percentage protein concentration. Experiment carried out at 39° in 0.07M phosphate (pH 7.3).

(2) *Experiments with vacuum dried heart muscle preparations*
Horse heart preparation (20 ml) yielded 832 mg powder on drying in the manner previously described. It was preserved in a desiccator for periods up to 4 weeks and resuspended in water (41.6 mg/ml) as required. For manometric experiments 0.05 ml of this suspension was used, while control runs were carried out with the corresponding amounts of undried material. The effect of phosphate concentration on this preparation is shown in Fig. 1. The results collected in Table 5 show that the activity of the preparation towards succinate is very much reduced by drying, when tested in 0.07M phosphate, and it is not reactivated by cytochrome c. Addition of denatured globin, however, increases the activity of the dried material to that obtained at the optimum phosphate concentration, and the addition of cytochrome c at this stage leads to a further increase in O₂ uptake. Cytochrome c, therefore, does not act here as an indifferent protein. An analogous oxidase

preparation from ox heart was less markedly affected by drying, probably owing to the presence of proteins which may function as denatured globin. In both cases, however, the effects produced by cytochrome c and by globin are additive. The results obtained with dried oxidase preparations in experiments of the type set out in Table 5 can vary considerably from one preparation to another and with the age of the preparation. Nevertheless, the distinction between the general effect of proteins and the specific effect of cytochrome c always remains clear.

Table 5 *Oxidation of succinate by heart-muscle preparations before and after drying. Effect of cytochrome c and denatured globin*

(Globin (0.3 ml 1.4%), 0.07M phosphate buffer, temp, 39°, pH, 7.3)

	O ₂ uptake (μl/15 min)		
	Horse heart		Ox heart
	Fresh	Dried	(dried)
Oxid + succ	78	2	62
Oxid + succ + cyt c	98	9	102
Oxid + succ + denat globin	193	80	145
Oxid + succ + denat globin + cyt c	220	174	252

(3) *Experiments with horse kidney preparations* The characteristic property of this type of preparation is that it oxidizes succinate or *p*-phenylenediamine very slowly unless cytochrome c is added, when the rate of O₂ uptake may increase at least 5 fold (Table 2). We have previously noted this marked effect of added cytochrome c upon kidney preparations and have suggested that it is due to a deficiency of cytochrome c (Keilin & Hartree, 1940). When the cytochromes in our kidney preparations are examined spectroscopically after reduction with succinate or Na₂S₂O₄, a feeble band (*a*) is seen together with a broad diffuse band (*b*₁), which occupies the same position as the two distinct bands (*b*) and (*c*) found in heart preparations. In the same paper we showed that it was possible to obtain heart preparations that were not deficient in cytochrome c and yet were virtually inactive in the aerobic oxidation of succinate unless soluble c was added. It is thus possible for c to be present in an unavailable form. In this connexion the results of Slater (1949) are of interest. He has found that if the acidification to pH 5.5 is avoided during the preparation of the kidney oxidase (see p 206) and replaced by high speed centrifuging as a means of concentrating the active material, a normal heart-type cytochrome spectrum can be observed on addition of succinate. However, the rate of oxidation of succinate by such material, which shows an optimal effect of [phosph], is also low in absence of added c, hence the c in the preparation must be largely inaccessible to other components of the catalytic system.

Experiments with our kidney preparation, under standard experimental conditions, are shown in Table 6. The distinction between the accelerating effects of cytochrome c and denatured globin is apparent from the fact that globin is ineffective in absence of added c.

Table 6 *The effect of various activating factors on the oxidation of succinate by a horse kidney preparation*

(Denatured globin (0.3 ml 1.4%), 4×10^{-4} M AlCl₃, 10^{-3} M methylene blue, 0.12 ml kidney preparation, 0.07M phosphate buffer, temp, 39°, pH, 7.3)

Factor added	O ₂ uptake (μl/30 min)
None	15
Denatured globin	22
AlCl ₃	21
Meth blue	54
Cyt c	111
Cyt c + globin	172
Cyt c + AlCl ₃	155

E *Effect of metals on the oxidation of succinate by different preparations*

Horecker, Stotz & Hogness (1939) have shown that low concentrations of salts of Al⁺⁺⁺, Cr⁺⁺⁺ and the rare earths markedly increase the rate of oxidation of succinate in presence of an ox-heart preparation. Swingle, Axelrod & Elvehjem (1942) found a similar activation of various tissue preparations by Ca⁺⁺ (see p 210) while Schneider & Potter (1943) have studied the activation of an unwashed liver preparation by Ca⁺⁺ and Al⁺⁺⁺.

(1) *Effect of Ca⁺⁺ and Al⁺⁺⁺* The influences of these metallic ions added separately on the aerobic oxidation of succinate by heart muscle preparations are quite different from those recorded by Schneider & Potter who found that, in liver extracts, Ca⁺⁺ alone activated the system and that Al⁺⁺⁺ merely abolished an initial lag in O₂ uptake. In Fig 3

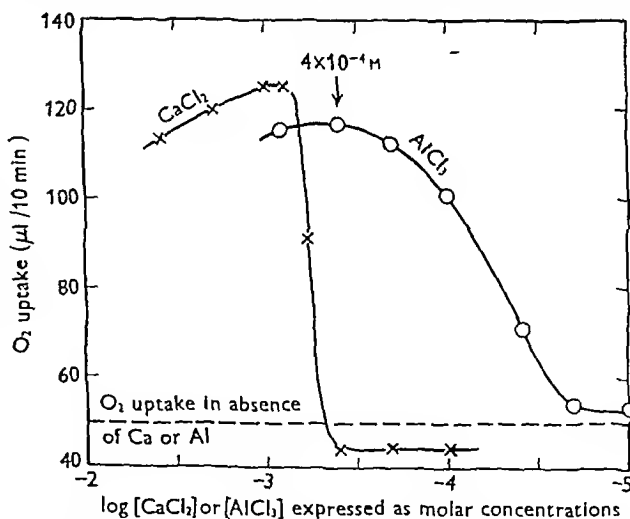


Fig 3 *Effect of different concentrations of CaCl₂ and AlCl₃ on the rate of O₂ uptake by 0.1 ml heart-muscle preparation in presence of cytochrome c and succinate 0.07M phosphate, pH 7.3, temp, 39°. Abscissae log [CaCl₂] or [AlCl₃] expressed as molar concentrations*

are shown the effects of various concentrations of CaCl₂ and AlCl₃ on the aerobic activity of a horse-heart preparation under the standard conditions. It was of interest to find that the concentration at which the effect of Ca⁺⁺ disappears

is also the minimum concentration at which a gelatinous precipitate of $\text{Ca}_3(\text{PO}_4)_2$ is formed in the manometer flask. In the case of Al^{+++} the minimum concentration could not be gauged as the precipitate forms very slowly at low Al^{+++} concentration. At $4 \times 10^{-4} \text{M}$, only Al^{+++} is effective and the activation by the ions was found not to be additive. Anaerobic experiments with methylene blue gave no useful results owing to co precipitation of the dye with the insoluble phosphate. The fact that the effects produced by $\text{Ca}^{++} + \text{Al}^{+++}$ and by globin are not additive suggests that their modes of action are similar. They both form precipitates in the manometric flasks, thus providing surfaces for possible adsorption, which corroborates the conclusion reached by Horecker *et al.* (1939), namely that the activation they obtained with Cr^{+++} and other ions arose from the formation of precipitates of heavy metal phosphates. The dried oxidase preparations are also markedly reactivated by Ca^{++} and Al^{+++} . Different results, however, were obtained when $\text{CaCl}_2 + \text{AlCl}_3$ were added to the oxidase preparation before drying. In such experiments, to 10 ml heart muscle preparation were added 0.43 ml each of 0.6M CaCl_2 and 0.6M AlCl_3 in order that the final concentration of the two salts should be $4 \times 10^{-4} \text{M}$ in the subsequent manometer experiments. After vacuum drying and resuspension of the powder in 10 ml water, the preparation was found to be completely inactive and further addition of Ca^{++} and Al^{+++} failed to reactivate it, whereas the control dried preparation behaved normally (Table 7).

Table 7 Activity of succinic system in horse-heart preparations dried with or without the addition of CaCl_2 and AlCl_3

		$(4 \times 10^{-4} \text{M CaCl}_2 \text{ and AlCl}_3, \text{ temp } 39^\circ, \text{ pH } 7.3)$	
		$\text{O}_2 \text{ uptake}$ ($\mu\text{l}/20 \text{ min}$)	
Oxid dried with Ca^{++} and Al^{+++} + succ + cyt c	0		
Oxid dried with Ca^{++} and Al^{+++} + succ + cyt c	0		
+ $\text{CaCl}_2 + \text{AlCl}_3$			
Oxid dried alone + succ + cyt c	17		
Oxid dried alone + succ + cyt c + $\text{CaCl}_2 + \text{AlCl}_3$	109		

Our results cannot be interpreted in the same manner as those of Swingle, Axelrod & Elvehjem (1942). By working with homogenates of unwashed tissues, these workers obtained evidence that the effect of Ca^{++} arises from its activation of an enzyme hydrolyzing coenzyme I, the latter being an indirect inhibitor of succinic dehydrogenase (see p 215). In fact we were able to show that a filtrate of boiled heart preparation was completely devoid of this coenzyme since it could not replace authentic coenzyme I in the reconstituted muscle lactic dehydrogenase system (Straub, 1940). Further arguments against such a possibility are (1) that heart-muscle preparations in optimal [phosph] are not affected by Ca^{++} since the latter merely raises the activity to the optimal level, and (2) no effect of Ca^{++} can be observed when using enzyme material from other sources or when experiments are carried out in phosphate free buffers (Table 8).

(2) *Effects of other metals* The influence of other metals on the activity of a heart preparation in phosphate and in glyoxaline buffers and on a pigeon preparation in phosphate buffer is summarized in Table 8. In no case was there any activation of heart preparations in glyoxaline buffer or of pigeon preparations in phosphate buffer. The activations produced by Ba^{++} , Fe^{+++} , Mn^{++} and Ce^{+++} resemble those obtained with Ca^{++} and Al^{+++} . Inhibition is always complete in the case of Cu^{++} , but variable in the case of Co^{++} . The formation of dense granular precipitates by Ce^{+++} , Co^{++} and Cu^{++} in glyoxaline accompanies complete inactivation. The lack of activation by metals of the pigeon preparation in phosphate buffer finds a parallel in the absence of any effect of proteins, whether native or denatured, under the same circumstances.

F Evidence for the existence of a carrier linking succinic dehydrogenase with cytochrome

Several workers claim to have demonstrated the existence of, or even to have isolated, a carrier linking succinic dehydrogenase with the cytochrome system. We shall now summarize the evidence

Table 8 Effect of metals on the succinic system in horse heart and pigeon breast muscle preparations

(Heart preparation (0.05 ml), 0.07 ml. pigeon preparation, 0.2 ml cytochrome c in all cases. Metals ($2 \times 10^{-3} \text{M}$) added as chlorides or sulphates, 0.07M buffer, temp, 39° , pH, 7.3)

Metal added	Preparation and buffer					
	Heart prep in phosphate		Heart prep in glyoxaline		Pigeon prep in phosphate	
	Rate*	Ppt †	Rate*	Ppt †	Rate*	Ppt †
None	(100)	—	(100)	—	(100)	—
Ba^{++}	171	I	96	‡	88	I
Ca^{++}	208	I	108	O	86	I
Ce^{+++}	181	I	0	D	85	I
Co^{++}	68	S	0	D	43	S
Cu^{++}	0	I	0	D	0	I
Fe^{+++}	174	I	86	§	94	I
Li^+	104	O	109	O	100	O
Mn^{++}	175	I	109	O	110	I

* Relative initial rates of O_2 uptake

† Key to letters and signs: O, no precipitate formed during experiment, I, immediate precipitate of gelatinous phosphate, S, slow formation of precipitate, D, slow formation of dense granular precipitate, ‡, slight crystalline precipitate of BaSO_4 , § precipitate of $\text{Fe}(\text{OH})_3$.

brought forward in support of these claims and re examine it in the light of our experiments

(1) The first enzyme preparation with properties suggesting the existence of a carrier was obtained by Hopkins, Lutwak-Mann & Morgan (1939) They washed a mince of pig heart with sodium chloride and lithium chloride solutions and extracted it with bile salts After precipitation of this extract with ethanol and extracting the precipitate with urea, a suspension was obtained which did not reduce cytochrome *c*, and which did not catalyze the oxidation of succinic acid by O_2 unless methylene blue was added Unfortunately, this treatment denatures the components *a*, *a₃* and *b* of cytochrome and inactivates the major part of the succinic dehydrogenase In fact, the Q_{O_2} of this preparation was about 12 whereas the Q_{O_2} of an active heart preparation lies between 500 and 700 It has been shown (Keilin & Hartree, 1940) that 10 min. contact with 50 % ethanol brings about the following changes in our heart muscle preparation (1) the cytochrome oxidase activity, as measured by its ability to oxidize *p*-phenylenediamine, is abolished, (2) cytochromes *a*, *a₃* and *b* are found, on spectroscopic examination, to be irreversibly modified, and (3) the succinic dehydrogenase activity, measured by the rate of O_2 uptake in presence of methylene blue, is reduced to about one third of its original value The behaviour of the preparation of Hopkins *et al* (1939) is thus best explained as being due to inactivation of the cytochrome system rather than to removal of an essential factor

(2) By ultracentrifuging a heart muscle preparation, Stern & Melnick (1939) obtained a preparation incapable of oxidizing succinic acid in presence of cytochrome *c*, although it contained an active succinic dehydrogenase, oxidizing the substrate by means of methylene blue, and an active cytochrome system as measured by the rate of oxidation of hydroquinone However, when the supernatant fluid was added to the ultracentrifuged preparation, a complete system was obtained which catalyzed the aerobic oxidation of succinic acid The supernatant fluid, according to them, contained 'a supplementary principle' or 'activator' which is a protein of molecular weight about 140,000 As we have mentioned previously (Keilin & Hartree, 1940), we were not in a position to repeat this experiment, but on centrifuging our heart preparation at a lower speed (13,000 r.p.m.) we obtained a sediment which was as active as the original material As any factor which remained in solution at the higher speed must also have remained at our lower speed it is impossible to reconcile the two experiments on the basis of the separation of a component The inactivation of Stern & Melnick's ultracentrifuged preparation cannot, therefore, be considered as being due to the separation of a

carrier It is probable that, under the influence of a high gravitational field, the preparation of Stern & Melnick underwent changes similar to those caused by drying or freezing No such effect was observed in the lower gravitational field of our experiment

We have already seen that marked activation of various types of enzyme preparation may be obtained with indifferent and denatured proteins That Stern & Melnick's results may find an explanation along the same lines appears probable as a result of our experiments with dried heart and kidney preparations We have shown (Table 5, second column of figures) that on drying a horse-heart preparation its catalytic activity towards succinic acid in presence of added cytochrome *c* is reduced to about 9 % of that of the original material In contrast to the inappreciable effect of cytochrome *c* on the dried preparation, the addition of denatured globin markedly raises the activity, and the further addition of cytochrome *c* at this stage more than doubles the activity so that it approaches 80 % of that of the fresh preparation The analogy with the results of Stern & Melnick becomes more striking when the activities of dried heart and kidney preparations are examined in presence of methylene blue, cytochrome and denatured globin The results obtained with these heart and kidney preparations are shown in Table 9 and Fig 4 respectively It is seen that the activation by cytochrome *c* in such cases is very small and considerably less than that by methylene blue In other words, a dried preparation catalyzes the oxidation of succinate by methylene blue (Fig 4, iv) more efficiently than by cytochrome *c* (iii) In absence of added cytochrome *c* globin has no effect on this system (i and ii), but the simultaneous addition of globin and cytochrome *c* leads to a very marked activation (v) Whereas the addition of methylene blue to this last system does not increase its catalytic activity, an increase is obtained when either cytochrome *c*, globin, or both are absent (ia, iia, iiia) Thus a non-specific and denatured protein appears to facilitate the reaction between dehydrogenase and the cytochrome system in the same way as the supplementary principle or activator of Stern & Melnick The latter cannot, therefore, be considered, without further evidence, as a specific component of the catalytic system in tissues which oxidize succinic acid.

(3) Straub (1942) treated an extract of pig-heart muscle with sodium cholate and ammonium sulphate and resuspended the resulting precipitate in phosphate buffer This 'cholate preparation' oxidized *p*-phenylenediamine, but not succinate unless methylene blue was added It thus contained both the cytochrome system and succinic dehydrogenase, but apparently a certain factor linking the two was missing A material which fulfilled this function

('SC factor') was obtained by heating a portion of the heart extract in alkaline solution. Straub considers the 'SC factor' to be an essential intermediate link in the cytochrome-succinic dehydrogenase system. This factor must be very different from the thermolabile protein 'activator' described by Stern & Melnick.

Table 9 *The effect of methylene blue and denatured globin on the succinic system in a dried horse heart preparation*

(Denatured globin (0.3 ml, 1.4%), 10^{-3} M methylene blue, dried oxidase, succinate, cytochrome c, 0.07M phosphate, temp, 39° , pH, 7.3)

Addition	O ₂ uptake (μ l/15 min)
None	11
Meth blue	58
Globin	160
Globin + meth blue	184

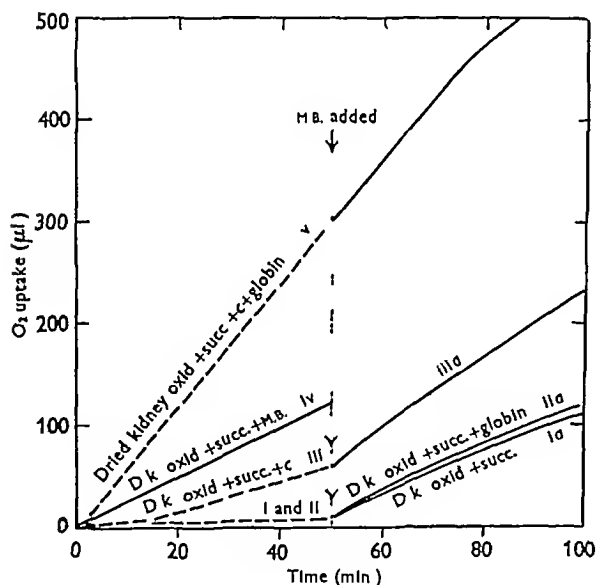


Fig 4 Effect of cytochrome c (0.2 ml, 1%), denatured globin (0.3 ml, 1.4%) and methylene blue (10^{-3} M) on the oxidation of succinate by a dried kidney oxidase preparation. Oxidase preparation equivalent to 0.1 ml of material before drying. 0.07M phosphate buffer, pH, 7.3, temp, 39° .

We isolated the cholate preparation of Straub strictly according to his description, and we obtained his 'SC factor' by incubation of the heart-muscle preparation for 30 min at pH 9 and 20° instead of at the higher temperature given in his paper which, in our hands, yielded a completely inactive material. Having confirmed Straub's results (Table 10, 1-4) we attempted to replace the 'SC factor' by substances which activated our enzyme preparations. Calcium phosphate was found to be much more efficient than the SC factor, whereas

denatured globin or Al^{+++} were completely ineffective (Table 10, 5-8). Such a striking difference between calcium phosphate and other activators was not observed with any of the enzyme preparations previously examined. The marked activating effect of Ca^{++} in this case is apparently due to precipitation as calcium salt of the residual cholate. The low activity in Exp. 1 can, therefore, be reasonably ascribed to an inhibition of the catalytic system by sodium cholate present in this preparation. A notable increase in the activity of the 'SC factor' was found after 3 days' storage at 5° , although the dehydrogenase activity of the cholate preparation

Table 10 *The effect of Ca^{++} and denatured globin on the succinic system prepared according to Straub (1942)*

(Cholate preparation (0.5 or 1.0 ml), 1.0 ml 'SC factor', 0.2 ml 1% cytochrome c, 0.04M sodium succinate, 0.3 ml $Ca_3(PO_4)_2$ gel (20 mg dry wt), 0.3 ml 1.4% denatured globin, 10^{-3} M methylene blue, temp, 39° , pH, 7.3)

	O ₂ uptake (μ l/10 min)	
	Tested same day using cholate prep (1.0 ml)	Tested 3 days later using cholate prep (0.5 ml)
1 Chol prep + cyt c + succ	7*	2
2 Chol prep + cyt c + succ + 'SC factor'	31†	92
3 'SC factor' + cyt c + succ	0	2
4 Chol prep + meth blue + succ	342	145
5 As 1 + $Ca_3(PO_4)_2$	232†	—
6 As 1 + denatured globin	2	—
7 As 2 + $Ca_3(PO_4)_2$	231	—
8 As 2 + denatured globin	33	—

* This system is not activated by 10^{-3} M $AlCl_3$, nor by increasing [phosph]

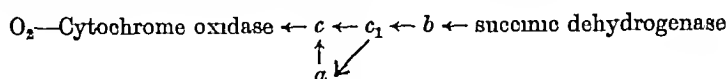
† O₂ uptake slows down after first 10 min

decreased during the same interval. This change coincides with an increased aggregation and precipitation of the proteins in the factor which may then act as a more efficient adsorbent of cholate. It is, therefore, probable that the 'SC factor' does not represent a specific link in the catalytic chain of the system oxidizing succinate, but that it merely serves to remove cholate from solution. On the other hand, since we obtained the 'SC factor' under conditions that were slightly different from those specified by Straub, further study will be required before the mechanism of its action can be definitely established (see also Slater, 1949).

(4) By fractionating an aqueous extract of minced ox or rabbit liver with ammonium sulphate Stoppani (1947) isolated a preparation which, although it contained both succinic dehydrogenase and the complete cytochrome system, was yet incapable of catalyzing the aerobic oxidation of

succinate even in presence of added cytochrome *c*. This material was precipitated from the liver extract within the range 0.2 saturation with ammonium sulphate. However, when the fraction which precipitated between 0.33 and 0.6 saturation was purified through calcium phosphate it was found to have a strong activating effect on the first fraction in the aerobic oxidation of succinate. One of the peculiarities of the first fraction as a succinic dehydrogenase was that it could utilize ferricyanide as H acceptor but not methylene blue. The activator was easily destroyed by heat and by acids and would appear to have been a protein. It could not be replaced by 10^{-5} M Ca^{++} or Al^{+++} or by different coenzymes.

In our attempts to repeat Stoppani's experiments, following his methods as closely as possible, we were



(arrows indicate direction of electron transfer)

unable to obtain fractions which showed a differentiation in the reactions with ferricyanide and methylene blue. In fact all fractions that we obtained between 0 and 0.2 saturation readily reduced methylene blue anaerobically in presence of succinate. On one occasion we were able to obtain from rabbit liver two fractions precipitated within the specified ammonium sulphate concentrations which resembled Stoppani's materials. Details of this experiment are set out in Table 11, where it is seen that the factor may be replaced by Ca^{++} or horse serum but not by Al^{+++} or denatured globin. We have used much higher concentrations of Ca^{++} and Al^{+++} than did Stoppani, which may well explain the discrepancy (cf Fig. 3).

Table 11 *Effect of Ca, Al and proteins on the succinic system prepared according to Stoppani (1947)*

(Enzyme preparation, rabbit liver (0.5 ml), 1 ml 'factor' (rabbit liver) 0.2 ml 1% cytochrome *c* (in all experiments), 10^{-3} M methylene blue, 0.005 M AlCl_3 , 0.005 M CaCl_2 , 0.14% denatured globin, 0.3 ml horse serum, 0.04 M sodium succinate, 0.07 M phosphate buffer, temp, 39° , pH, 7.3)

	O_2 uptake ($\mu\text{L/hr}$)
1 Enzyme + succinate	3
2 'Factor' + succinate	3
3 Enzyme + 'factor' + succinate	124
4 As 1 + Ca^{++}	107
5 As 1 + Al^{+++}	8
6 As 1 + serum	87
7 As 1 + denatured globin	21
8 As 1 + meth. blue	76

Our experiments suggest that the activating material isolated by Stoppani contains no essential link in the catalytic system oxidizing succinate, but comes within the category of indifferent proteins

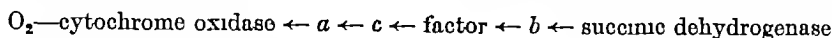
which can have a marked effect upon our heart-muscle and kidney preparations.

(5) Yakushiji & Okunuki (1940) have described a haem protein of haemochromogen type under the name cytochrome *c*₁ and consider it to be a catalytic intermediate in the cytochrome system. They repeatedly washed an isoelectric precipitate of a heart preparation with acetate buffer, pH 4.6, before suspending it in phosphate at pH 7.5 and found that, on reduction with succinate + HCN, it showed an absorption band at $552\text{ m}\mu$. This pigment, which can be extracted from heart preparations in a soluble form, will reduce cytochromes *a* and *c*. These authors suggest that it acts as an oxidation-reduction carrier between cytochrome *b* and cytochromes *a* and *c*.

However, since *c*₁ can only be detected or isolated after treatments which have an adverse effect upon the activity of the complete succinic system (e.g. sodium cholate or ether) the possibility that it is an artifact derived from other haem-proteins (see p. 214) must be seriously considered. On the other hand, the function postulated for this substance is very similar to that put forward by Slater (see below) for a labile haem protein detected by him in heart muscle preparations, and it is possible that the two 'factors' may be closely related.

(6) While studying the effect of BAL (2,3-dimercaptopropanol) on the succinic dehydrogenase-cytochrome system, Slater (1948, 1949) has obtained a heart-muscle preparation exhibiting interesting properties. Although this preparation failed to catalyze the aerobic oxidation of succinic acid, it showed an almost unimpaired activity both of succinic dehydrogenase, as shown by the rate of oxidation of succinate in presence of methylene blue, and of the cytochrome system estimated by the oxidation in presence of cytochrome *c* of ascorbic acid, hydroquinone or *p*-phenylenediamine. On addition of succinate to such a preparation, cytochrome *b* becomes permanently reduced, whereas the other cytochrome components remain in the oxidized state, an effect resembling that observed on addition of urethane to a normal preparation (Keilin & Hartree, 1939, 1940). On addition of $\text{Na}_2\text{S}_2\text{O}_4$, the reduced components of cytochrome (*a*, *a*₃, *b*, *c*) appear normal, although the total protohaematin content of the preparation, determined by addition of pyridine plus $\text{Na}_2\text{S}_2\text{O}_4$ and measurement of the intensity of the haemochromogen band at $548\text{--}560\text{ m}\mu$, is 20% below that of the heart preparation before treatment with

BAL Slater's interpretation of these results is that the heart preparation contains a BAL-labile factor which is an intermediate in the electron transfer from cytochrome *b* to cytochrome *c* and which is probably a haematin derivative. The system may be represented as follows



The preparation obtained by Slater shows some resemblance to two preparations that we obtained by incubating heart muscle oxidase either at pH 5.0 or at a higher pH in presence of pancreatin (Keilin & Hartree, 1940). In all cases there appears to be a lack of interaction between the succinic dehydrogenase on the one hand and the cytochrome system on the other. We suggested that the impairment of the aerobic succinoxidase activity could be explained, either by loss of a factor intermediate between dehydrogenase and cytochrome *c* or by an irreversible change in the colloidal structure of the preparation, with a consequent decrease in the mutual accessibility of the catalysts in the system.

Although we cannot exclude the possibility of the existence of several further intermediate links between the known components of the succinic dehydrogenase cytochrome oxidase system, we have seen that much of the evidence is inconclusive, and so far only that brought forward by Slater appears to be well founded upon experimental evidence.

DISCUSSION

The tissue preparations used for the study of the system catalyzing the aerobic oxidation of succinic to fumaric acid consist of colloidal suspensions of protein aggregates. In addition to the complete succinic dehydrogenase cytochrome system these preparations also contain fumarase together with small concentrations of catalase and certain dehydrogenases (e.g. malic and lactic), they are, however, free from haemoglobin and coenzymes I and II. Being completely devoid of substrate, they have no O_2 uptake, show cytochromes in a completely oxidized state and do not reduce methylene blue anaerobically. After addition of sodium succinate the cytochromes rapidly become reduced and on aerating the mixture they re-oxidize only to become again reduced when aeration ceases. The preparation will now take up O_2 rapidly and will reduce methylene blue anaerobically.

Spectroscopically the preparation shows the cytochrome components *a*, *a*₃, *b* and *c* (Keilin & Hartree, 1939). It also contains other forms of haematin which were previously described as free haematin (Keilin, 1925), but which we now consider to be present as one or more haematin-protein derivatives (Keilin & Hartree, 1947*b*). That such compounds are not visible spectroscopically may be due to their

diffuse absorption bands. However, on treating these preparations with $\text{Na}_2\text{S}_2\text{O}_4$ and pyridine, the extra haematin becomes spectroscopically visible as pyridine haemochromogen, the α band of which is clearly seen obliterating the space between the α bands of cytochromes *b* and *c*.

Since the activity of the complete succinic system can be studied both spectroscopically and manometrically, we are able to localize the actions of a number of inhibitory or activating factors and to determine the mechanism of their action. Further analysis of this system can be made by independent study of the activities of its two portions. Thus the activity of succinic dehydrogenase can be determined either anaerobically by the rate of reduction of methylene blue, or aerobically, by the rate of O_2 uptake in presence of methylene blue and of cyanide which inhibits the cytochrome oxidase. The activity of the cytochrome system can readily be followed manometrically by the rate of oxidation of substances which reduce cytochrome *c* directly, i.e. without the need of specific enzymes. Such substances are ascorbic acid, cysteine, phenols and aromatic amines.

Whereas some factors act on one or other of the known components of the system others affect either some, as yet undetermined, intermediate components, or the colloidal structure of the preparation which assures the mutual accessibility of the components. In any attempt to analyze the complex systems represented by our colloidal preparations it is important to determine not only the point at which the factor reacts but also its mode of action. Even if we confine our attention to inhibitors we find that in some cases the mode of action is easily demonstrated, while in others it requires a more thorough investigation. Thus the powerful, instantaneous and perfectly reversible inhibition of the system by HCN, NaN_3 or H_2S is due to their combination with the trivalent Fe of cytochrome oxidase (*a*₃), while the effect of CO depends upon its ability to combine with oxidase Fe in the divalent state. All these reactions can be followed spectroscopically (Keilin & Hartree, 1939). On the other hand, malonate (Quastel & Wooldridge, 1928), oxaloacetate (Das, 1937) and pyrophosphate (Dixon & Elliott, 1929; Leloir & Dixon, 1937) act as competitive inhibitors of succinic dehydrogenase. The relative affinity of this enzyme (*E*) for substrate (*s*) and inhibitor (*i*) determined experimentally and expressed as a partition constant $K = [Ei][s]/[Es][i]$ is about 13 for malonate, 23 for pyrophosphate and 850 for oxaloacetate. Spectroscopic study of enzyme preparations shows that these inhibitors prevent the reduction of all components of cytochrome by succinate.

On the other hand the mechanisms of the inhibition of this system by coenzyme I and by sodium diethyldithiocarbamate (DDC) are less direct and require a more careful consideration. The inhibition of aerobic oxidation of succinate by coenzyme I was first recorded by Potter (1939). We confirmed this effect and found a marked inhibition at coenzyme concentrations as low as 0.5×10^{-5} – 2.5×10^{-5} M. At first sight this inhibition could be ascribed to a direct reaction of coenzyme with some reactive groups of the dehydrogenase. However, a study of this reaction under varying conditions revealed the following mechanism (Keilin & Hartree, 1940). Since heart muscle preparations contain fumarase and malic dehydrogenase, part of the fumarate arising from the oxidation of succinate is transformed to malate which, in presence of coenzyme, is oxidized to oxaloacetate. The latter, being neither reduced nor decarboxylated at an appreciable rate, acts as a competitive inhibitor of succinic dehydrogenase. In fact, if the concentration of oxaloacetate reaches only 0.2 % of that of the succinate it would already inhibit the O_2 uptake by > 50 % (see p. 214).

The study of the inhibition by DDC is of special interest. This substance is chiefly known as a reagent for the colorimetric estimation of copper and will act as an inhibitor of reactions catalyzed both by Cu^{++} ions and by copper protein enzymes such as polyphenol oxidase. The inhibition by this substance of the aerobic oxidation of succinate at first suggested the possibility that a copper derivative participated in the reaction. However, a further study of this reaction clearly showed that the inhibition was not due to DDC itself but to its oxidation product tetraethyldithiocarbamyl disulphide (TDD) $(C_2H_5)_2NCS \cdot SS \cdot SCN(C_2H_5)_2$ derived from DDC through an oxidation catalyzed by the cytochrome system, since DDC reduces cytochrome c (Keilin & Hartree, 1940). In fact it was found that while DDC inhibited succinate oxidation only after it had become oxidized to TDD, in concentrations as low as 5×10^{-6} and 2×10^{-5} M, inhibited O_2 uptake immediately to the extent of 70 and 100 % respectively.

Since the demonstration by Hopkins & Morgan (1938) that the activity of succinic dehydrogenase depends upon the integrity of certain —SH groups in the enzyme, the mechanism of action of a number of inhibitors has been explained by their ability to combine with —SH groups or to oxidize them (see Potter & DuBois, 1943; Slater, 1949).

The activity and behaviour of the succinic system show considerable variations both with the type of tissue used and with the species of animal from which it was obtained. The effects of different factors, such as phosphate concentration, native and denatured proteins and salts of a number of metals,

were tested both on fresh enzyme preparations and on vacuum-dried and frozen samples.

The study of the influence of phosphate concentration on the activity of a heart preparation has demonstrated a relationship between activity and the degree of colloidal dispersion. We have not attempted to express the degree of dispersion in any quantitative form, but have regarded the time taken for the proteins in a preparation to flocculate spontaneously and settle out under gravity as a rough measure of the degree of dispersion. Thus the diluted heart-muscle extract described on p. 206, which remains homogeneous for several hours, is regarded as completely dispersed. Maximum dispersion and enzyme activity are obtained in 0.15 M phosphate (pH 7.3) with an increasing flocculation and falling off in activity as the concentration deviates from this figure. When the total salt concentration remains constant and [phosph] is varied, an activating effect of phosphate is detectable and the occurrence of an optimum concentration is apparently due to two opposing influences: peptization and salting out. In our kidney preparation the former influence is absent, hence the activity increases continuously with diminishing [phosph]. A point of certain interest is that fully active heart preparations can be obtained in absence of phosphate, glyoxaline replacing phosphate during the extraction from the muscle and in the manometers.

We have shown that, contrary to the views of Biró & Szent Györgyi jun (1946), ATP does not activate, but slightly inhibits the system oxidizing succinic acid. If any link exists between this system and ATP it must be indirect and may depend upon catalytic systems not present in our preparations. Moreover, even if the synthesis of ATP is linked with the oxidation of succinate it does not logically follow that ATP should have any effect upon the activity of the succinic dehydrogenase-cytochrome system.

The importance of colloidal structure for the activity of the complete system is clearly demonstrated by the fact that when [phosph] is reduced to a suboptimal value, e.g. 0.07 M, the addition of denatured globin or of Ca^{++} or Al^{+++} brings the activity of the system to the level shown at the optimum [phosph] (Table 3). These additions give rise to bulky precipitates on which the enzyme preparation becomes adsorbed although similar, but less marked, effects are observed with proteins that remain in solution (Fig. 2 and Table 4). The effect of denatured globin is even more striking in the cases of dried heart and dried kidney preparations (Table 5 and Fig. 4). Where the metals do not form bulky precipitates (e.g. in glyoxaline buffer) no activation is obtained while a lack of activation in phosphate buffer by either proteins or metals is observed if the enzyme preparation already consists

of a bulky flocculent precipitate as in the case of pigeon breast muscle (see Keilin & Hartree, 1947b, table 2, also Table 8 of this paper)

We have seen that the activation of the system by proteins generally and by cytochrome *c* may be of the same order of magnitude. This naturally raises the question of the exact function of added *c* in our experiments. That it acts as an oxido-reduction catalyst and not as an indifferent protein is clearly demonstrated by the following observations.

(1) When cytochrome *c* is denatured by treatment with alkali it becomes autoxidizable and, at the same time, catalytically inactive (Keilin & Hartree, 1940). Such material fails to accelerate the O_2 uptake of a heart preparation oxidizing succinate at a concentration equal to the optimal concentration of native *c* (Fig. 2).

(2) Unlike indifferent proteins or metallic ions, cytochrome *c* markedly increases the rate of oxidation of succinate by fresh kidney preparations (Table 6).

(3) The above proteins and ions may activate some preparations to an equal or even greater degree than does cytochrome *c*. However, the effects upon O_2 uptake induced by the indifferent activators and by *c* are additive or even synergic (Table 5).

(4) Dried enzyme preparations from horse heart or kidney show a very low catalytic activity towards succinic acid. The reactivation of a dried horse-heart preparation by denatured globin is usually greater than that produced by cytochrome *c*. However, when both substances are added simultaneously, the resulting activity is markedly greater than the sum of the activities in presence of each of these substances acting separately. The behaviour of such preparations can, however, vary quite widely (see p. 209). A dried kidney preparation (Fig. 4), which fails to oxidize succinate, cannot be reactivated by proteins. Some reactivation is obtained with *c*, but methylene blue is more effective. On the other hand, denatured globin and cytochrome *c* added together will produce a very striking increase in the activity of the dried preparation, and bring it almost to the level of activity of the fresh material with the same additions.

It was previously shown (Keilin & Hartree, 1940, 1945) that the catalytic activity of cytochrome *c* within intact cells and tissues or their colloidal extracts is much greater than that of *c* added in soluble form. The experiments described in this paper lend considerable support to our view that the high activity of the succinic system in such extracts is due largely to the retention within the colloidal particles of the favourable spatial organization of catalysts characteristic of the cell. On such a hypothesis the much lower catalytic activity of added *c* is understandable. Moreover, the loss in activity of a tissue preparation treated in different

ways, even when it can be made good by the addition of *c*, is less likely to be due to loss of endogenous *c* than to changes in colloidal structure which diminish the accessibility of endogenous *c* to other components of the system. The changes resulting from the various treatments can be reversed, and the system as a whole reactivated by addition of factors having an influence on the colloidal structure, such as proteins, ions yielding insoluble phosphates, and changes in [phosph]. An explanation of this reactivation can only be proposed in very general terms. Thus the factors may be responsible either for reorientation of the components, especially of endogenous cytochrome *c*, within the colloidal particles or for providing new reactive surfaces to which catalysts, in particular exogenous *c*, may become anchored. On these grounds the wide variations in response to these factors from one preparation to another are understandable.

The more or less complete loss of accessibility of cytochrome *c* may show different degrees of reversibility. In the extreme case of the dried kidney preparation the removal of *c* from the sphere of influence of other components is almost irreversible since it is impossible to reactivate the succinic system by addition of Ca^{++} or proteins, and, furthermore, added *c* cannot be utilized catalytically unless such factors are also added. This preparation is of special interest since it resembles those obtained by several workers in their attempts to demonstrate the existence of new intermediary links between succinic dehydrogenase and cytochrome *c*. Such preparations share the property of being more strongly activated by methylene blue than by cytochrome *c*, and of being very highly activated when the latter is supplemented by another factor isolated from the same tissue. Although we have seen that it is not necessary to postulate the absence from the dried kidney preparation of a specific link in the succinate oxidizing system, one cannot rule out the possibility that one or more additional links between known components of the succinic dehydrogenase cytochrome system do, in fact, exist. However, we have shown that the evidence brought forward is inadequate in all cases with one possible exception. The only reasonable evidence is that of Slater (1948), who postulates a specific link, probably of haematin nature, acting as an oxido-reduction carrier between cytochromes *b* and *c*. So far this conclusion is based solely upon an irreversible destruction of the factor and not upon its separation from an active preparation.

The activating effects that we have obtained may be summarized as follows. Marked changes in catalytic activity are associated with the introduction of bulky precipitates. If such precipitates are formed by addition of metallic salts giving gelatinous phosphates, or of proteins, an increase in activity may

be observed. If, on the other hand, precipitates are formed from the enzyme material itself there is generally a loss of activity (e.g. freezing or drying). Lost activity can frequently be restored by formation of precipitates of the activating type.

Treatments which reduce the activity by modifying the colloidal structure may (1) make endogenous cytochrome *c* unavailable or (2) make both endogenous and added *c* unavailable to the rest of the system. It is because the latter effects may often be reversed by indifferent proteins that claims for the existence of new 'links' in the succinic system have been made. Similarly, activation by Ca^{++} of our preparation is not due to an accelerated removal of coenzyme I.

Previous evidence for new 'links' and for the function of Ca^{++} was in each case based upon one type of preparation which was studied under a fixed set of conditions. Our results, however, show that a slight modification of these conditions may, through changes in colloidal structure, lead to fundamentally different results.

We may fittingly conclude by quoting from an earlier paper on the same subject (Keilin & Hartree, 1940): 'The succinic dehydrogenase cytochrome system even in the cell-free colloidal preparations behaves as a true respiratory system of the cell, showing a high catalytic activity and being affected by all inhibitors in the same way and to the same degree as the normal respiration of intact cells. The efficiency of the system depends, however, not only on the integrity of its components, but also on that of the colloidal structure which supports them and assures their mutual accessibility.'

SUMMARY

1. A study of the succinic dehydrogenase cytochrome system in various types of tissue extract has been made, with special reference to the effect of colloidal structure on enzyme activity.

2. Heart-muscle preparations show optimum activity in 0.15 M-phosphate, at lower phosphate concentrations addition of proteins or certain metallic ions raises the activity to the optimum level. In this respect insoluble denatured proteins are the most effective. The activity of these preparations falls considerably after freezing, but it can be restored by addition of cytochrome *c*. The pronounced loss of activity that accompanies vacuum drying can be fully reversed only by adding both cytochrome *c* and either proteins or certain metal salts.

3. Kidney preparations are feebly active unless cytochrome *c* is added. They are virtually inactive

after vacuum drying, but can be restored to full activity by addition of both cytochrome *c* and protein, although each added separately has very little effect. The activity of these preparations falls as the buffer concentration is increased, there is no optimum concentration.

4. The activation by metals is obtained in phosphate buffer, where gelatinous precipitates are formed, but not in glycylglycine buffer.

5. It is considered that the catalysts in the colloidal particles, as in the intact cells, are more or less rigidly held together within a framework which ensures their mutual accessibility and a consequent high catalytic activity. Any change in colloidal structure will therefore modify the overall activity without necessarily removing or destroying any individual catalyst.

6. The inhibitory effects of freezing, drying, extreme [phosph], are intimately bound up with the formation from the enzyme material of flocculent precipitates and a consequent decrease in inter-availability. Activation, however, arises when flocculent precipitates of foreign materials (proteins, gelatinous phosphates) are formed in the medium. Such material provides new surfaces upon which reorientation can take place. Since the pigeon preparations already consist of flocculent material they do not undergo activation.

7. It has been clearly demonstrated that added cytochrome *c* acts in these experiments solely as an oxidoreduction catalyst and not as a protein affecting the colloidal structure.

8. The various claims to have demonstrated the existence of new 'links' in the succinic system (comprising dehydrogenase, cytochromes *b*, *c*, *a* and a_3) have been examined in detail. All are shown to be based upon insufficient evidence except that of Slater (1948), who has produced reasoned arguments in favour of an oxidoreduction catalyst linking *b* and *c*.

9. Since our preparations are devoid of coenzyme I the view that its activation by Ca^{++} arises from an accelerated decomposition of this coenzyme, as is the case with fresh homogenates, is untenable. Furthermore, Ca^{++} is ineffective with certain types of tissue preparation.

10. Two types of factor (activating or inhibiting) can be distinguished. They may function either by reacting specifically with a catalyst in the system or by affecting the colloidal structure of the enzyme material. Activation of the latter type has often been misinterpreted as evidence for additional carriers or 'links' in the succinic dehydrogenase-cytochrome system.

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The Component Acids of some Seal Blubber and Liver Fats

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The fatty oils of seals have received little detailed investigation, but their general analytical characteristics as recorded in the literature (cf Halden & Grun, 1929) reveal considerable variations in the blubber oils, iodine values from 122 to 163 having been reported. This unusually wide range is reflected in the two specimens of seal blubber oil which have so far been examined in greater detail, namely, a commercial sample (probably of Newfoundland origin) with an iodine value of 135.8 (Burke & Jasperson, 1944) and the blubber fat of a grey Atlantic seal (*Halichoerus grypus*) caught off the coast of Pembroke, with an iodine value of 162.2 (Hilditch & Pathak, 1947). The chief differences observed in the detailed analyses of these two seal oils consisted in variation in the proportions of unsaturated C_{16} acids, unsaturated C_{18} acids, and unsaturated C_{22} acids.

When Prof A N Worden of the University College of Wales, Aberystwyth (who had very kindly put material from the grey Atlantic seal at our disposal earlier), informed us that similar specimens from two common seals (*Phoca vitulina* L.) were to be available, it was felt desirable to undertake similar examination of their blubber and liver lipids in

order to add to the somewhat meagre knowledge of the fatty acids present in the fats of members of this family. Prof Worden was accordingly good enough to secure for us samples of blubber and of liver from two common seals taken off the Norfolk coast in July 1947, the lipids from which were extracted and examined in our laboratory.

METHODS

The tissues (blubber or liver) were cut into small pieces and extracted exhaustively with hot acetone until no further lipid was removed. The material left after removal of the acetone was taken up in light petroleum (b.p. 40–60°), the solution separated from the water which had also been entrained by the acetone, and the lipids recovered. The liver lipids contained appreciable quantities of phosphatides as well as glycerides, and these were separated by keeping their acetone solutions at 0° for several days, when the phosphatides were precipitated. The component fatty acids of the blubber oils and (so far as the quantities available permitted) of the liver glycerides and phosphatides were determined by the methods used in our earlier work (Hilditch & Pathak, 1947) on the blubber and liver oils of the grey Atlantic seal. These are briefly as follows.

(a) The mixed fatty acids from the oil were first partly resolved by crystallization from acetone and from ether at

low temperature Crystallization from 10% solution in acetone at -60° left in solution nearly all of the polyethenoid acids of the C_{20} and C_{22} series, with some oleic and much of the hexadecenoic acid, in one instance further quantities of the more unsaturated acids were obtained by subsequent recrystallization from acetone at -60° of the insoluble and soluble portions obtained in the first crystallization. In all instances the acids finally left insoluble in acetone at -60° were crystallized from 10% solutions in ether at -40° , when material of very low iodine value was deposited, leaving in solution a concentrate of mainly monoethenoid acids, accompanied by small amounts of saturated acids and polyethenoid acids of the C_{20} series.

(b) Each group of acids thus obtained was separately converted into methyl esters, which were fractionally distilled in a vacuum through an electrically heated and packed column. From the iodine values and equivalents of the resulting ester fractions the compositions of the latter were calculated by methods which have been described elsewhere (Hilditch, 1947). The mean unsaturation, expressed by the fractional number of hydrogen atoms short of saturated, e.g. -2.0 (monoethenoid), was determined by interpolation or extrapolation from the respective ester fractions in each group from which the mean equivalent of each of the homologous ester groups (C_{16} , C_{18} , C_{20} and C_{22}) follows. In this manner the fatty acid composition of each separated group of fatty acids, and thence that of the original seal blubber or liver lipid, was finally obtained.

RESULTS

The animals from which the lipids were obtained were I, a female about 2 or 3 weeks old, 915 mm long (snout to tip of tail), girth at axilla 510 mm, II, a yearling male, 1020 mm long, girth at axilla 735 mm. The specimens will be referred to as I and II throughout this paper.

Blubber oils

The blubber fats were almost wholly glyceridic and contained no phospholipids separable by deposition from acetone at 0° . They were pale yellow oils with the following analytical characteristics:

	Oil of seal I	Oil of seal II
Iodine value	140.0	145.4
Saponification equivalent	289.0	292.3
Free fatty acid (as oleic) (%)	0.2	0.1
Unsaponifiable matter (%)	0.1	0.3

Component acids of blubber oil from seal I The mixed fatty acids (186 g) were crystallized first from acetone at -60° , the deposited acids then being further crystallized from ether at -40° .

Group	Description	Weight		Iodine value
		(g)	(%)	
A	Insoluble in ether at -40°	25.7	13.8	4.9
B	Soluble in ether at -40°	75.4	40.6	99.6
C	Soluble in acetone at -60°	84.9	45.6	218.1

The ester fractionation data for the methyl esters of each of these three groups of acids are summarized in Table 1, and the values allotted to

Table 1 *Fractionation of methyl esters of seal I blubber oil acids A, B and C*

Fraction	Weight (g)	Saponification equivalent	Iodine value
Methyl esters of acids A			
A1	2.89	217.6	0.4
A2	3.73	269.0	1.9
A3	3.50	269.8	2.1
A4	3.56	271.5	2.8
A5	3.77	271.9	3.0
A6	3.61	282.6	3.6
A7	3.54	313.5*	23.9
	24.60		
Methyl esters of acids B			
B1	2.82	254.2	52.2
B2	4.20	266.8	85.9
B3	4.66	266.9	88.1
B4	3.87	273.0	88.4
B5	5.11	280.7	88.6
B6	5.30	294.3	88.6
B7	5.54	295.1	89.0
B8	4.99	296.2	89.2
B9	3.98	296.8	92.3
B10	3.73	305.7	92.9
B11	3.25	313.1	117.4
B12	3.05	321.3*	152.8
	50.50		
Methyl esters of acids C			
C1	4.12	247.1	92.1
C2	3.28	268.4	99.7
C3	3.90	270.1	103.2
C4	4.68	275.7	109.7
C5	4.08	281.7	120.5
C6	3.86	283.3	123.0
C7	3.92	285.2	125.0
C8	3.52	287.1	151.0
C9	4.09	311.1	236.7
C10	6.16	330.9	330.7
C11	4.58	335.1	380.6
C12	4.50	338.6	373.5
C13	3.10	346.8*	334.7
	53.79		

* Equivalents of esters (freed from unsaponifiable matter) A7, 303.7, B12, 314.6, C13, 342.7

Table 2 *Equivalents, iodine values and mean unsaturation employed in calculating the composition of unsaturated methyl ester fractions from groups B and C*

Methyl esters of acids	Saponification equivalent	Iodine value	Mean unsaturation
Group B			
C ₁₄	240.0	105.8	- 2.0
C ₁₆	268.0	94.8	- 2.0
C ₁₈	295.9	90.1	- 2.1
C ₂₀	321.6	173.8	- 4.4
C ₂₂	—	—	—
Group C			
C ₁₄	240.0	105.8	- 2.0
C ₁₆	267.9	99.5	- 2.1
C ₁₈	294.0	172.8	- 4.0
C ₂₀	319.0	270.3	- 7.0
C ₂₂	343.0	407.3	- 11.0

the various homologous groups of unsaturated C_{14} , C_{16} , C_{18} , C_{20} and C_{22} acids in each series are shown in Table 2

The percentage composition (by weight) of each group of acids was then calculated, and therefrom the composition of the total fatty acids of the blubber oil (Table 3)

Component acids of blubber oil from seal II In this case the first crystallization of the mixed fatty acids (235.5 g) of the oil from acetone at -60° caused 90.9 g (iodine value 70.1) to be deposited, and left in solution 144.6 g (iodine value 202.8). The latter acids, on repetition of this process (acetone at -60°), deposited 40.7 g (*D*, iodine value 84.1) and left in solution 103.9 g (*E*, iodine value 250.2). The acids deposited in the original crystallization were also recrystallized from acetone at -60° , when there were left in solution 20.1 g (*C*, iodine value 145.3), and 70.8 g (iodine value 47.0) were deposited and then crystallized from ether at -40° . This led to the separation of 32.5 g

(*A*, iodine value 2.6), leaving in solution in the ether 38.3 g (*B*, iodine value 84.4)

There were thus obtained the following five groups of acids, each of which was methylated and fractionally distilled in the usual manner

Group	Weight		Iodine value
	(g)	(%)	
<i>A</i>	32.5	13.8	2.6
<i>B</i>	38.3	16.3	84.4
<i>C</i>	20.1	8.5	145.3
<i>D</i>	40.7	17.3	84.1
<i>E</i>	103.9	44.1	250.2

It is perhaps unnecessary to devote the space required for the presentation of the detailed ester fractionation data (as in Table 1) and it is, therefore, proposed only to quote the fatty acid compositions finally obtained for each of the five groups of acids (% w/w) and, therefrom, of the total fatty acids of the seal II blubber oil (Table 4)

Table 3 *Component acids in groups A, B, C and in the whole seal I blubber oil*

Acid	Component acids in groups			Fatty acids in the whole oil		
	<i>A</i>	<i>B</i>	<i>C</i>	(% w/w)	(% by mol)	Mean unsaturation
	(13.8%)* (%)†	(40.6%)* (%)†	(45.6%)* (%)†			
Myristic	9.7	1.6	0.9	2.4	2.9	—
Palmitic	65.0	3.2	—	10.3	11.1	—
Stearic	16.5	0.6	—	2.5	2.4	—
Arachidic	2.2	—	—	0.3	0.3	—
Unsaturated C_{14}	Trace	1.9	4.7	2.9	3.6	- 2.0
Unsaturated C_{16}	1.6	28.5	30.4	25.7	27.8	- 2.1
Unsaturated C_{18}	3.3	51.8	24.3	32.6	31.8	- 2.7
Unsaturated C_{20}	1.2	12.3	15.2	12.1	10.8	- 5.9
Unsaturated C_{22}	—	—	24.4	11.2	9.3	- 11.0
Unsaponifiable	0.5	0.1	0.1	—	—	—

* Group as % (w/w) of total oil † Component acids as % (w/w) of group

Table 4 *Component acids in groups A-E and in the whole seal II blubber oil*

Acid	Component acids in groups					Fatty acids in whole oil	
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>	(% w/w)	(% by mol)
	(13.8%)* (%)†	(16.3%)* (%)†	(8.5%)* (%)†	(17.3%)* (%)†	(44.1%)* (%)†		
Myristic	6.5	2.5	2.0	1.5	1.0	2.2	2.7
Palmitic	58.3	5.8	3.3	7.3	—	10.6	11.4
Stearic	28.7	2.1	1.4	0.1	—	4.4	4.3
Arachidic	2.3	—	—	—	—	0.3	0.3
Unsaturated C_{14}	Trace	2.5	3.4	2.0	2.6	2.2	2.7
Unsaturated C_{16}	1.0	14.1	32.1	28.1	24.2	20.8	22.7
Unsaturated C_{18}	2.1	66.2	33.2	49.7	25.2	33.7	33.2
Unsaturated C_{20}	0.7	6.5	22.7	11.2	19.4	13.6	12.4
Unsaturated C_{22}	—	—	1.5	—	27.2	12.2	10.3
Unsaponifiable	0.4	0.3	0.4	0.1	0.4	—	—
Mean unsaturation of							
Unsaturated C_{14}	- 2.0	- 2.0	- 2.0	- 2.0	- 2.0	- 2.0	—
Unsaturated C_{16}	- 2.0	- 2.0	- 2.0	- 2.0	- 2.1	- 2.1	—
Unsaturated C_{18}	- 2.0	- 2.0	- 2.5	- 2.0	- 3.0	- 2.4	—
Unsaturated C_{20}	- 2.0	- 5.0	- 7.5	- 4.5	- 8.0	- 7.2	—
Unsaturated C_{22}	—	—	- 11.0	—	- 11.0	- 11.0	—

* Group as % (w/w) of total oil † Component acids as % (w/w) of group

Liver lipids

Seal I The moist liver tissue (338 g) yielded to acetone a total quantity of 14.6 g of lipids soluble in light petroleum, and contained 24.9 g of water. The dry liver tissue thus contained 16.4% of lipids (4.3% on the moist tissue). These were dissolved in ten times their weight of acetone, and the solution left at 0° for some weeks, when 3.4 g of sticky dark-brown material (phosphatides) were precipitated, leaving in solution 11.2 g of glyceridic oil.

Phosphatides (crude) thus formed about 23% of the liver lipids. The amount available was too small to permit any examination of the fatty acids present. The material contained 4.7% P and 1.9% N (P:N ratio, 1:1.1).

About 77% of the seal liver lipids consisted of glycerides which, however, contained 18.5% of unsaponifiable matter. A portion (7.25 g) of the liver glyceride mixed fatty acids, from which unsaponifiable matter had been removed, was crystallized first from acetone at -60°, the deposited acids being further crystallized from ether at -40°. From the iodine values of the three fractions so obtained an approximate estimate of the saturated, monoethenoid and polyethenoid acids present was derived (assuming average iodine values of 90 and 350 respectively for the monoethenoid and polyethenoid material).

	Weight		Equivalent	Iodine value
	(g)	(%)		
(i) Insoluble in ether at -40°	1.77	24.4	270.7	14.2
(ii) Soluble in ether at -40°	1.48	20.4	289.3	88.9
(iii) Soluble in acetone at -60°	4.00	55.2	312.1	267.0

from which is calculated

	Approximate component acids (expressed as % of total acids)		
	Saturated	Monoethenoid	Polyethenoid
(i)	20.5	3.9	—
(ii)	0.3	20.1	—
(iii)	—	17.7	37.5
	20.8	41.7	37.5

The component acids of the seal I liver glycerides consisted, therefore, of about 20% saturated acids and about 40% each of monoethenoid and polyethenoid fatty acids.

Seal II The moist liver tissue (409 g) yielded to acetone 43.6 g of lipids soluble in light petroleum, i.e. 10.7% on the moist tissue. The moisture content of the latter is unfortunately uncertain, but the lipid content of the dry tissue must have been of the order of at least 50%. Thus the liver of the common seal II had a much higher fatty content than either that of the common seal I or that of the

gray seal studied earlier (Hilditch & Pathak, 1947), and the subsequent examination showed that the increase in lipid content was wholly due to liver glycerides. The crude liver lipids (43.6 g), after keeping in acetone solution at 0° for some weeks, deposited 2.3 g of crude phosphatidic material, and left in solution 41.3 g of glyceridic oil.

Crude phosphatides thus formed only 5% of the total liver lipids, and, again, were too small in amount for their fatty acids to be examined. The crude material contained 2.4% P and 1.0% N (P:N ratio, 1.06:1).

The crude glycerides contained 9.7% of unsaponifiable matter, so that the actual glyceride content of the liver lipids was about 86% (as compared with about 63% in the liver lipids of seal I). After removal of the unsaponifiable matter, the quantity of glyceridic mixed fatty acids (32.6 g) available was sufficient for approximate analysis by ester fractionation (Table 5). The acids were first resolved into two fractions by crystallization from acetone at -60°.

Group	Description	Weight		Iodine value
		(g)	(%)	
A	Insoluble in acetone at -60°	16.3	50.0	67.7
B	Soluble in acetone at -60°	16.3	50.0	299.4

Table 5 Fractionation of methyl esters of seal II liver glyceridic fatty acids A and B

Fraction	Weight (g)	Saponification equivalent	Iodine value
Methyl esters of acids A			
A1	2.15	269.3	15.5
A2	1.77	276.2	23.6
A3	2.58	283.9	36.3
A4	3.21	293.2	60.1
A5	2.84	299.5	72.5
A6	3.20	316.3*	90.6
	15.75		
Methyl esters of acids B			
B1	1.81	268.1	87.3
B2	2.46	298.4	160.9
B3	2.84	314.5	294.5
B4	3.05	326.3	328.4
B5	2.44	338.8	373.7
B6	3.34	349.4*	300.6
	15.94		

* Equivalents of esters (freed from unsaponifiable matter) A6, 310.8, B6, 344.3

The unsaturation of the homologous esters in group B was estimated to be as follows:

Methyl esters of acids	Saponification equivalent	Iodine value	Mean unsaturation
C ₁₆	268.0	94.8	- 2.0
C ₁₈	294.5	151.0	- 3.5
C ₂₀	318.0	319.2	- 8.0
C ₂₂	343.0	407.3	- 11.0

The fatty acid composition (% w/w) of groups A and B, and thence those of the total fatty acids in the seal II liver glycerides, based on the ester-fractionation data in Table 5, are shown in Table 6

At first sight it would appear that, as might be expected, the compositions of the fatty oils from the two common seals, taken at the same time in the same area, are not very different. Actually the only

Table 6 Component acids in groups A and B, and in the whole seal II liver glycerides

Acid	Component acids in groups		Fatty acids in whole oil	
	A	B		
	(50.0%)* (%)†	(50.0%)* (%)†	(%, w/w)	(%, by mol)
Myristic	0.3	0.2	0.2	0.3
Palmitic	22.1	0.7	11.4	12.9
Stearic	15.4	—	7.8	7.9
Arachidic	0.3	—	0.1	0.1
Unsaturated C ₁₆	6.8 (-2.0)‡	10.4 (-2.0)‡	8.6 (-2.0)‡	9.8
Unsaturated C ₁₈	40.4 (-2.0)	15.1 (-3.5)	27.9 (-2.4)	23.7
Unsaturated C ₂₀	14.3 (-2.0)	32.9 (-8.0)	23.7 (-6.2)	22.5
Unsaturated C ₂₂	—	40.4 (-11.0)	20.3 (-11.0)	17.8
Unsaponifiable	0.4	0.3	—	—

* Group as % (w/w) of total oil
† Component acids as % (w/w) of group
‡ Mean unsaturation

DISCUSSION

Seal blubber oils

The data obtained in the course of the present work are most usefully considered in conjunction with the detailed component acid figures which have been given for other seal blubber oils, namely, the grey seal (Hilditch & Pathak, 1947) and the mixed specimen of seal oil probably from Newfoundland or the north eastern Atlantic seaboard (Burke & Jasperson, 1944). The percentages by weight of the component acids of all four oils are collected in Table 7.

A significant difference is in the somewhat greater proportion of unsaturated (hexadecenoic) C₁₆ acids in the oil of seal I, which is offset by minor decreases in unsaturated C₁₈, C₂₀ and C₂₂ acids and in stearic acid as compared with the oil of seal II. The mean unsaturation of the C₂₀ acids in the oil of seal II is definitely greater than in the oil of seal I. Apart from these points, however, the two oils are not dissimilar: the chief component is the unsaturated C₁₆ group (about 33% of the total acids, and largely oleic acid) followed by about 21–25% of hexadecenoic acid, saturated acids, especially palmitic acid, are remarkably constant not only in these two oils, but in all four seal oils quoted in Table 7.

Table 7 Component acids (% w/w) of seal blubber oils

	Common seal I	Common seal II	Grey seal	Newfoundland (?) seal
Iodine value of oil	140.0	145.4	162.2	135.8
Acid				
Myristic	2.4	2.2	3.7	5.1
Palmitic	10.3	10.6	10.5	10.7
Stearic	2.5	4.4	2.0	1.3
Arachidic	0.3	0.3	—	0.6
Unsaturated C ₁₄	2.9	2.2	1.6	1.8
Unsaturated C ₁₆	25.7	20.8	15.5	10.5
Unsaturated C ₁₈	32.6	33.7	30.8	39.6
Unsaturated C ₂₀	12.1	13.6	16.5	17.6
Unsaturated C ₂₂	11.2	12.2	18.1	10.6
Unsaturated C ₂₄	—	—	1.3	2.1
Mean unsaturation of				
Unsaturated C ₁₆	-2.1	-2.1	-2.2	-2.1
Unsaturated C ₁₈	-2.7	-2.4	-2.7	-2.4
Unsaturated C ₂₀	-5.9	-7.2	-5.7	-5.6
Unsaturated C ₂₂	-11.0	-11.0	-10.6	-9.3
Unsaturated C ₂₄	—	—	-11.0	-10.9

It is only when comparison is made with the earlier results that it appears that the component acids of different seal blubber oils may vary to a considerable degree. The grey seal belongs to a different genus of the family, whilst the biological source of the Newfoundland oil cannot of course be given with any certainty. Whilst species differences may well account for at least part of the difference in composition between the blubber oils of the grey seal and the common seal, it is noteworthy that in these four oils we have a marked range of variation in some of the component acid groups, and that this is most evident in the hexadecenoic acid group, which lies between the extremes of about 10 and 26% of the total acids. In three of the four oils the unsaturated C_{18} (mainly oleic) acid content is similar, but in the Newfoundland oil, with lowest unsaturated C_{18} acid content, the percentage of the unsaturated C_{18} group rises to nearly 40% of the total acids. Similarly, whilst the combined content of unsaturated C_{20} and C_{22} (and C_{24}) acids in the blubber oils of the common seal is about 23–26%, this rises in the grey seal oil to 36%, in the Newfoundland oil the unsaturated C_{20} acid group is similarly high, but the unsaturated C_{22} group is still at the level observed in the common seal oils. Apart from the C_{20} acids in seal oil II (already mentioned), the mean unsaturation of the various series of acids appears to remain fairly constant in all the oils, irrespective of the variations in their proportion to which attention has just been drawn.

In the only other group of marine mammalian blubber oils—those of the Balaenidae—which have been studied to any extent, there are fairly well-defined differences in the proportions and average unsaturation of the different groups of component acids as between different genera and species of whales, but a number of independent specimens of Antarctic whale oil taken in different years show closely similar fatty acid compositions. So far as the few instances discussed above go, the conclusion may be hazarded that the blubber oils produced by different species of seal vary more widely in composition between themselves than those produced by different groups of the whale family. The present results may perhaps indicate further that the blubber fats of one and the same species of seal tend to exhibit more variation in composition than those from a single species of whale. This, again, may be connected with the diet of the seal, which is likely to be much more variable than that of the whale.

It is accordingly not easy to assign any very general characteristics to the component acids of seal blubber oils, except that their content of saturated acids is usually about 18% (10–11% palmitic acid), that unsaturated C_{18} (mainly oleic) acid forms about 33% of the total acids, and that the rest is made up of unsaturated C_{16} acids (about

15–25%) and polyethenoid acids of the C_{20} and C_{22} series (23–30%, mean unsaturation of the C_{20} acids about –5 to –6 H, and of the C_{22} acids –10 to –11 H).

Seal liver lipids

The liver tissue of the seal contains only a low proportion of lipids, which consist of glycerides and phosphatides in varying proportions. The liver of the common seal II apparently contained more lipid (glycerides) than the average, and in this instance we were able to make an ester fractionation analysis of the liver-glyceride fatty acids. Comparative figures for the liver lipids of the common seal and of the grey seal are collected in Table 8.

Table 8 Composition of seal liver lipids

	Common seal I	Common seal II	Grey seal
Total lipid content of liver (moist) (%)	4.3	10.7	2.8
Composition of liver lipids (% w/w)			
Phosphatides (crude)	23	5	29
Glycerides	63	86	50
Unsapoifiable matter	14	9	21
Approximate composition of liver glyceride fatty acids (% w/w)			
Saturated	20	20	37
Monoethenoid	40	38	46
Polyethenoid	40	42	17

The composition of the liver lipids thus appears to be somewhat variable, with a ratio which perhaps in normal seal livers is of the order of two or three parts of glyceride to one of phosphatide. The component acids of the liver glycerides of the grey seal which we examined earlier (Hilditch & Pathak, 1947) were much less unsaturated than those of the corresponding blubber oil or of the liver glycerides of the common seal which we have now studied, and contained much less polyethenoid acids and nearly twice as much saturated acids as either of the latter. The detailed component acid figures for the liver and blubber glycerides of the common seal II are compared in Table 9.

Table 9 Component acids of the liver and blubber glycerides of common seal II

Acid	Liver (% w/w)	Blubber (% w/w)
Myristic	0.2	2.2
Palmitic	11.4	10.6
Stearic	7.8	4.4
Arachidic	0.1	0.3
Unsaturated C_{14}	—	2.2 (–2.0)*
Unsaturated C_{16}	8.6 (–2.0)*	20.8 (–2.1)
Unsaturated C_{18}	27.9 (–2.4)	33.7 (–2.4)
Unsaturated C_{20}	23.7 (–6.2)	13.6 (–7.2)
Unsaturated C_{22}	20.3 (–11.0)	12.2 (–11.0)

* Mean unsaturation

The liver glycerides contained considerably less hexadecenoic acids, somewhat less oleic and polyethenoid C_{18} acids, and considerably more polyethenoid C_{20} and C_{22} acids, the degree of unsaturation of the unsaturated components was similar in both liver and blubber glycerides. No comparable data are available for the liver and blubber glycerides of the whale or any other marine mammal, whilst similar comparisons in a number of fish species seem to indicate that no generalization can yet be made. Thus, in the sturgeon (Lovern, 1932b) and groper (Shorland & Hilditch, 1938) there is less fat in the liver than in the peritoneal cavity and pancreas (sturgeon) or the head (groper), but the component acids of liver and depot fats are similar in composition in the respective fish. This also obtains in the tunny (Lovern, 1936), in which the liver and flesh contain about the same proportion of fat. On the other hand, in halibut and turbot (Lovern, 1932a, 1937) the liver is the main fat depot, and the flesh contains but little fat. In both of these instances the unsaturated C_{16} acid contents of the liver glycerides are much higher, and the amount of polyethenoid C_{20} and C_{22} acids much lower, than in the corresponding flesh fats. This lack of correlation stands in contrast to the fats of land animals in which (Hilditch & Shorland, 1937) the liver glycerides are distinguished by definitely lower contents of stearic acid, and higher contents of hexadecenoic and of polyethenoid acids of the

C_{20} and C_{22} series, in comparison with the corresponding depot fats

SUMMARY

1 The composition of the blubber and liver lipids from two specimens of the common seal (*Phoca vitulina* L.) has been investigated.

2 The component acids of the blubber glycerides of the two animals were not dissimilar, but differed considerably from that of the grey seal (*Halichoerus grypus*). These differences are probably due partly to difference in species, but it also appears likely that differences in the food ingested by individual seals may be responsible to some extent for variations in the fat laid down.

3 The liver lipids of seals also appear to differ in amount and in their composition. One of the two common seal livers examined was exceptionally rich in glycerides as compared with phosphatides. Contrary to what was observed in the grey seal, the liver glycerides of the common seal are very similar in composition to its blubber oil.

4 So far, no correlation has been discernible between the composition of the liver glycerides and the glycerides of the flesh, head or other tissues of marine animals which may function as fat depots.

It is a pleasure once more to offer our thanks to Prof A N Worden and to Dr L Harrison Matthews for again providing us with interesting experimental material of authentic origin.

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The Action of Trypsin on Insulin

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In previous studies (Butler, Dodds, Phillips & Stephen, 1948) it was found that the action of trypsin on insulin is comparatively slight. It appeared likely that the slow action observed was due to a small proportion of chymotrypsin (about 0.5%) known to be present in the trypsin, and it was doubtful if there was any residual effect which might be ascribed to trypsin itself. In some further

experiments with the same materials it was found that the extent of the action was rather variable, and appeared to depend on several factors, e.g. the method of preparing the insulin solution, the age of the solution at the time of enzyme addition. The possibility that trypsin might cause a slight hydrolysis of insulin without decreasing its potency has led us to re-examine its action, using eventually

a purified trypsin kindly given us by Drs J H Northrop and M Kunitz, and a sample of insulin which had been several times recrystallized

EXPERIMENTAL AND RESULTS

The effect of trypsin on insulin solutions prepared in several ways

These experiments were carried out as described in a previous paper (Butler *et al* 1948), using the same trypsin, and an insulin concentration of 5 mg/ml. The insulin solutions were made up as indicated in Table 1. The extent of hydrolysis certainly varied with the method of preparation of the solution, but in most cases was comparatively small in the first 24 hr, and only became considerable after that period. This was probably due to the slow action of the chymotrypsin present on the insulin, which converts the latter into products upon which trypsin can act. Initial treatment with alkali, apparently, makes the insulin more susceptible to attack under these conditions.

Action of purified trypsin

The action of the purified enzyme on the commercial insulin was found, as might be expected, to be appreciably less especially in long periods than that of the trypsin used above, but a real residual action still remained. Table 2 shows the results obtained in two distinct experiments. In both cases there is an increase of non protein nitrogen (NPN) amounting to about 7% in 1 day and 12% in 2-3 days, and the amino N increases at the same time to about 10%, which would signify a quite appreciable splitting of

about 6 peptide bonds/insulin unit of 12,000. At the end of the experiments, the pH of the solutions was adjusted to 5.4 and the precipitates which formed were filtered off and dried. Their insulin potency, kindly determined for us by Boots Pure Drug Co. Ltd., was 56-59% of that of the original insulin. It is evident that in the long continued action of trypsin there had been considerable loss of potency.

Action of purified trypsin on recrystallized insulin

There remains some doubt whether the non protein N formed comes from insulin or extraneous substances. The insulin used was Boots zinc insulin, potency 22.8 units/mg. This was found to give a single sharp peak in the electrophoretic diagram. It was recrystallized four times, the first three by the method of Romans, Scott & Fisher (1940), and the last time by dissolving in dilute acetic acid and adding NaOH to pH 4.5. It should be pointed out that, according to a recent paper by Lens (1948), this procedure may result in the formation of some denatured material. The results obtained by digesting this insulin (5 mg/ml) with 135×10^{-4} units of the purified trypsin in 100 ml are shown in Table 3A. A similar control experiment without any trypsin present is shown in Table 3B. It is evident that the change in NPN in the presence of trypsin was not much greater than that in a similar buffer containing no trypsin. The insulin, however, lost a rather greater amount of potency in the presence of the trypsin than in its absence. The potency of the recovered material from the trypsin solution after 72 hr was greater than that after 48 hr, thus may be connected with the fact that a smaller proportion of the insulin was actually

Table 1 *Tryptic action on insulin solutions prepared in various ways*

(Non protein N (NPN) expressed as % of total N)

A		B		C		D		E	
Time (hr)	NPN (%)	Time (hr)	NPN (%)	Time (hr)	NPN (%)	Time (hr)	NPN (%)	Time (hr)	NPN (%)
0	—	0	7.6	0	7.3	0	9.7	0	11.6
17.5	9.7	23	14.7	18	10.9	23	13.6	18	17.5
44.5	19.2	46	18.0	42	44.7	48	45.6	42	23.2

A Insulin mixed with phosphate buffer, pH 7.6, before adding enough alkali to bring it into solution. Trypsin added at once.
 B Same as A, but trypsin added 70 hr after making up solution.
 C Insulin dissolved in 0.02N NaOH and same quantity of phosphate buffer then added. Trypsin added at once.
 D Same as C, but trypsin added 21 hr after making up solution.
 E Same as A.

Table 2 *Action of purified trypsin on insulin*

(Digest contains 5 mg insulin/ml in M/24 phosphate buffer, pH 8.2)

Trypsin 44×10^{-4} units in 30 ml			Trypsin 72×10^{-4} units in 50 ml		
Time (hr)	NPN (%)	Amino N (%)	Time (hr)	NPN (%)	Amino N (%)
0	5.5	4.4	0	6.6	4.4
3	5.7	—	23	14.4	9.3
21	12.5	—	47	18.9	9.5
44.5	12.5	—			
68.5	17.2	10.0			

N precipitated at pH 5.4, 82%

Potency of precipitate 13.4 units/mg

N precipitated at pH 5.4, 84.5%

Potency of precipitate 12.7 units/mg

Table 3 *Comparison of changes in digest with those in buffer alone*

A Action of purified trypsin on 4 times recrystallized insulin (25°)				
Time (hr)	0	48		72
N (% of T N)* in trichloroacetic filtrate (N P N)	4 0	7 6		11 1
N (% of T N) in isoelectric filtrate	—	6 4		11 5
Amino N (% of T N) in recovered insulin	4 4	6 3		5 8
Amide N (% of T N) in recovered insulin	9 9	8 4		9 0
Mean potency of precipitate (units/mg)	22 96	15 6		18 7
Limits of error, P=0 95	21 01-25 09	13 69-17 71		15 89-22 13
B Change of insulin with time in buffer solution, pH 8 4 (25°)				
Time (hr)	0	24	48	72
(a) N (% of T N) in trichloroacetic filtrate (N P N)	4 9	6 2	8 6	10 0
Potency of precipitate (units/mg)	22 9	—	—	20 6
Limits of error, P=0 95	—	—	—	18 5-22 9
(b) N (% of T N) in isoelectric filtrate	0 0	2 2	3 2	4 8
Amide N (% of T N) in recovered insulin	9 3	9 4	9 5	8 3

* T N =Total nitrogen

recovered in the former case. There was a slight increase in the percentage of amino N in the recovered insulin from trypsin, which may indicate the breakage of one or two peptide bonds. The results shown in lines (a) and (b), Table 3B, were obtained in separate experiments. The N P N values obtained in this experiment seem rather high, they are quoted because the potency of the insulin recovered from the buffer after 72 hr. was also determined. A re-determination of the N P N in the trichloroacetic acid filtrate after 72 hr. (exp. b) gave a value only about 3% greater than the N in the isoelectric filtrate. It is evidently difficult to reproduce the N P N formed in buffer solutions after long periods of time.

Dr K. O. Pedersen of Uppsala has kindly examined in the ultracentrifuge the products of trypsin action dissolved in a buffer solution containing Na_2HPO_4 (0.10M) and NaH_2PO_4 (0.05M). The sedimentation constant of the bulk of the material was found to be the same as that of untreated insulin (Pedersen, 1948), but a spreading of the sedimentation curves indicated that the material had become less homogeneous. The possibility that the degradation of insulin in the buffer solution was due to the contamination of the insulin by a proteolytic enzyme was considered. No evidence of the existence of the latter could be obtained when insulin was added to the haemoglobin substrates suggested for the assay of trypsin and pepsin by Anson (1938), but the concentration necessary to produce the effect with insulin could be too small to detect by the latter method.

Since trypsin exerts an amidase activity on certain amides (Bergmann, Fruton & Pollock, 1939; Schwert, Neurath, Kaufman & Snoke, 1948), and since insulin contains 8.9% of its nitrogen as amide N (Chibnall, 1942), we have also attempted to find if any of the latter is liberated by trypsin. The amide N was determined in a conventional manner (see Sanger, 1945), but no significant diminution was observed in trypsin-treated insulin.

Purification of insulin by treatment with trypsin

If, unlike other proteins, insulin is resistant to trypsin, it should be possible to purify it to some extent, at least, by tryptic digestion. A crude insulin preparation, kindly given to us by Boots Pure Drug Co. Ltd., had a potency of 8.2 units/mg. This material (0.5 g.) was dissolved in 85 ml.

of phosphate buffer, pH 7.8, and treated with crystalline trypsin (500×10^{-4} units) for 19 hr. at 25°. The pH was then adjusted to 5.2 with 0.1N HCl and the precipitate filtered off (dry wt. 59 mg.). Its potency, as determined by Boots, was 17.0 units/mg., i.e. the potency of the insulin had been doubled, but there was a considerable loss, since only 1000 units were recovered from an initial 4000. It is possible that purified trypsin would give a more satisfactory recovery, and that trypsin action may be a useful step in the purification of trypsin-resistant proteins.

DISCUSSION

Every increase in the purity of the enzyme preparation and of the insulin has resulted in a decrease of the action observed, and under the best conditions the change produced is slight. The increase in non-protein nitrogen in the presence of trypsin is of the same order as that in the buffer alone. There is, however, a slight increase in the free amino nitrogen of the recovered insulin and the potency of the recovered material is certainly appreciably less. It is therefore possible that the trypsin breaks one or two bonds in the insulin molecule, per unit of mol. wt. 12,000, without any non-protein peptide being liberated. The ultracentrifuge also shows a change in the nature of the recovered insulin. Although its mean sedimentation constant is unchanged, there is a greater spread of sedimentation rate, indicating an increased heterogeneity of the material. This might be due to a slight denaturation affecting the shape of the molecules and the way in which the submolecules aggregate.

SUMMARY

1. The action of trypsin on insulin has been re-examined. Purified trypsin has less action than the trypsin originally used.

2. Using four times recrystallized insulin and purified trypsin, the non-protein nitrogen formed is not much greater than that produced in the buffer.

alone, but in the presence of trypsin the physiological potency is diminished to a somewhat greater extent than in its absence. Ultracentrifugal examination of the recovered insulin shows it to have become less homogeneous than the original.

3 The action of trypsin on insulin is thus slight, but the possibility of the hydrolysis of one or two peptide bonds, with a diminution of potency of 10–20%, is not excluded.

One of us (J A V:B) holds the Courtauld Research Fellowship in this Institute. In addition our thanks are due to Courtaulds Ltd for a grant in aid of this investigation, to Imperial Chemical Industries Ltd for financial assistance, to Sir J Drummond, F R S, and to Dr W A Broom of Boots Pure Drug Co Ltd, for assays of the insulin preparations, to Drs J H Northrop and M Kunitz for purified trypsin, to Dr K O Pedersen for the ultracentrifugal examination of some of the products, and to Mrs I D R Goodwin for technical assistance.

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Studies in the Biochemistry of Micro-organisms

80 THE COLOURING MATTERS OF *PENICILLIUM ISLANDICUM* SOPP PART 1 1,4,5-TRIHYDROXY-2-METHYLANTHRAQUINONE

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(Received 30 July 1948)

Penicillium islandicum Sopp is a species included by Thom (1930) in the Funiculose series of the division of the *Penicillia* known as the *Biverticillata-symmetrica*. The species was first described and named by Sopp (1912). He isolated it in Norway from a mouldy specimen of skyr received from Reykjavik, Iceland. Skyr* is a bacterially soured milk peculiar to Iceland, and is somewhat similar in nature to Bulgarian yogurt. A number of other strains have been isolated subsequently in different parts of the world. Colonies of *P. islandicum* are characterized by their three coloured appearance, 'a green conidial zone near the margin and progressively overgrown with red orange to red hyphae in the central areas, reverse at first orange to sordid yellow orange shades, later becoming rich red shades' (Thom, 1930). We are engaged in a study of this complex mixture of colouring matters, and it is the purpose of the present communication to describe the isolation of one of them and its identification as the hitherto undescribed 1,4,5-trihydroxy-2-methylantraquinone.

* Thom (1930, p 466), in a description of *P. islandicum* Sopp, says 'species found on the Island of Skyr'. Sopp's (1912, p 162) original description states 'Der Pilz wurde auf isländischem Skyr gefunden'.

The new colouring matter, which was isolated by solvent extraction from five different strains of *P. islandicum* grown on Czapek-Dox 5% glucose solution, was obtained in yields of 3% of the dried mycelium of strain 1036. The total yield of the fat-free complex mixture of colouring matters was rather remarkable. It amounted to 20% of the dried mycelium. The nature of the other colouring matters is being investigated.

The new colouring matter forms large, dark red, lustrous plates or leaflets, m.p. 218°. It has the molecular formula $C_{15}H_{10}O_5$, contains one methyl group attached to carbon, no methoxyl group, and forms a triacetate, m.p. 208°, and a trimethyl ether, m.p. 161°, mol. wt. 314, 320. It is not soluble in aqueous sodium carbonate, but dissolves readily in sodium hydroxide giving a deep violet solution. Its solution in cold concentrated sulphuric acid is bright purple red in colour with a fiery red fluorescence. Its general behaviour is that of a polyhydroxyanthraquinone.

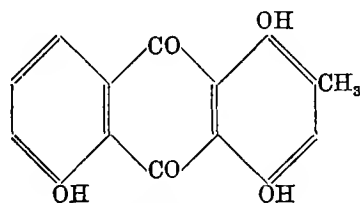
Its molecular structure as 1,4,5-trihydroxy-2-methylantraquinone was established as follows. Since its molecular formula is $C_{15}H_{10}O_5$, and it contains one methyl group and three hydroxyl groups (formation of a triacetate and a trimethyl ether), it

appeared probable that it was a trihydroxymethyl-anthraquinone. This hypothesis was confirmed by converting it, by mild oxidation with manganese dioxide and concentrated sulphuric acid, into the known compound, cynodontin (V). Cynodontin, 1,4,5,8-tetrahydroxy-2-methylanthraquinone, was first isolated by Raistrick, Robinson & Todd (1933a) from laboratory cultures of *Helminthosporium cynodontis* Marignoni and *H. euschlaenae* Zimmermann and later by the same workers (Raistrick, Robinson & Todd, 1934) from *H. avenae* Eidam. They established its constitution as (V) and this was confirmed by its synthesis by Anslow & Raistrick (1940b). Hence it follows that the *Penicillium islandicum* colouring matter must be one of the four possible 1,4,5-trihydroxy- β -methylanthraquinones of structures (I), (II), (III), (IV). Of these, (III) and (IV) are known compounds.

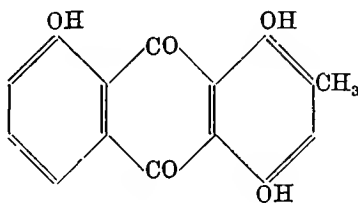
comparison of the *Penicillium islandicum* colouring matter with helminthosporin left no doubt that they were not identical, although they give colour reactions with sodium hydroxide and cold concentrated sulphuric acid which are almost indistinguishable from each other.

Compound (IV) has been synthesized by two different methods. Graves & Adams (1923) state that, on heating, (IV) sublimes at 250–260°. Keimatsu & Hirano (1930) give the m.p. of (IV) as 253–254°, its triacetate as 215° and its trimethyl ether as 206.5–207°. The corresponding melting points for the *P. islandicum* colouring matter are 218°, 208° and 161°. It is thus clear that the *P. islandicum* colouring matter is not identical with (IV).

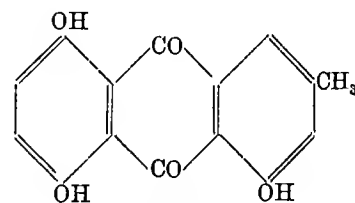
Hence it follows that the *P. islandicum* colouring matter must have either structure (I) or (II). This conclusion was confirmed and a choice made



(I)

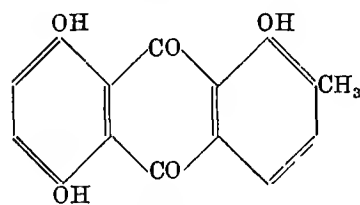
P. islandicum product

(II)

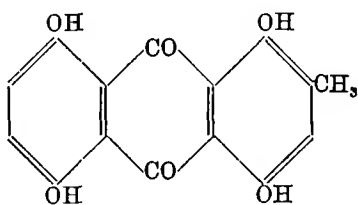


(III)

Helminthosporin

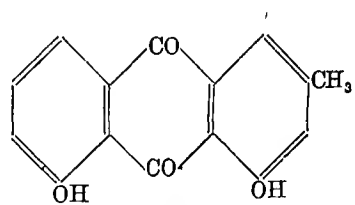


(IV)



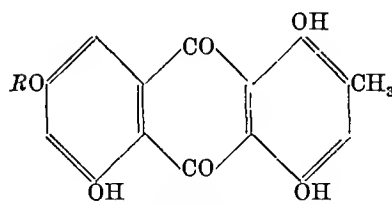
(V)

Cynodontin

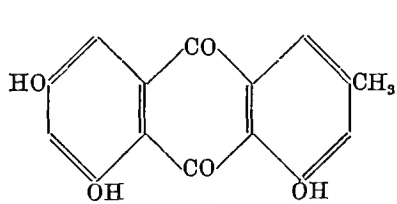


(VI)

Chrysophanic acid

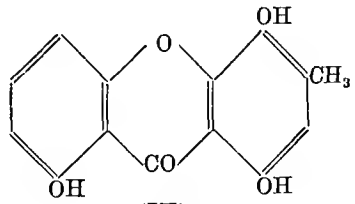


(VII)

 $R = H$, Catenarin $R = CH_3$, Erythroglaucon

(VIII)

Frangula emodin



(IX)

Ravenelin

Compound (III) is helminthosporin which was isolated from cultures of *Helminthosporium gramineum* Rabenhorst by Charles, Raistrick, Robinson & Todd (1933), also from *H. cynodontis* Marignoni (Raistrick *et al.* 1933a), and *H. catenarium* Drechsler and *H. tritici-vulgaris* Nisikado by Raistrick *et al.* (1934). Its molecular constitution, (III), was established by analytical means by Charles *et al.* (1933), and this was confirmed by its synthesis by Graves & Adams (1923) and by Raistrick, Robinson & Todd (1933b). Direct

between (I) and (II) by the following method. The *P. islandicum* colouring matter was reduced with concentrated hydriodic acid and red phosphorus in glacial acetic acid. The resulting anthranol, on oxidation with chromic acid in acetic acid solution, yielded chrysophanic acid (chrysophanol), the structure of which, (VI), was established by synthesis by Naylor & Gardner (1931). Of the structures (I), (II), (III) and (IV) only those which could give chrysophanic acid, (VI), by removal of any one hydroxyl group are (I) and (III). Compound (III)

is helminthosporin. Hence it follows that the *P islandicum* colouring matter must be (I), i.e. 145-trihydroxy-2-methylanthraquinone.

It is interesting to note that the *P islandicum* colouring matter is closely related structurally, not only to the mould colouring matters helminthosporin and cynodontin, but also to catenarin (VII, $R=H$) and erythroglaucon (VII, $R=CH_3$), which are respectively the 7-hydroxy and 7-methoxy derivatives of the *P islandicum* colouring matter. Catenarin was isolated from laboratory cultures of *Helminthosporium catenarium* Drechsler, *H velutinum* Link and *H tritici-vulgaris* Nisikado by Raistrick *et al* (1934). Its molecular constitution (VII) was established by Anslow & Raistrick (1940a), and this was confirmed by synthesis by the same investigators (Anslow & Raistrick, 1941). Erythroglaucon was isolated by Ashley, Raistrick & Richards (1939) from a number of species in the *Aspergillus glaucus* series, and its molecular constitution was established by Anslow & Raistrick (1940a). They also showed that, on reduction with hydriodic acid and red phosphorus in glacial acetic acid followed by oxidation of the resulting anthranol with chromic acid, catenarin (VII) yields *Frangula* emodin (VIII). We have stated previously that the *Penicillium islandicum* colouring matter (I), on similar treatment, yields chrysophanic acid (VI). It will be noted that in each case it is the hydroxyl group in the ortho position to the methyl group which is removed.

A further example of the close structural relationship between many mould colouring matters is afforded by ravenelin. This substance, 148-trihydroxy-3-methylxanthone (IX), was isolated from *Helminthosporium ravenelin* Curtis and *H turcicum* Passerini, and its molecular constitution established by Raistrick, Robinson & White (1936). Its structural relationship to the *Penicillium islandicum* colouring matter (I) is obvious.

Two trihydroxymethylanthraquinones of unknown molecular constitution having properties similar to those of 145-trihydroxy-2-methylanthraquinone have been described. Nataloemodin (m.p. 214–215°, triacetate, m.p. 204°) was obtained by Léger (1902, 1905) by degradation of nataloin. He found that nataloin prepared from Natal aloes, on treatment with alkaline sodium peroxide, yielded nataloemodin monomethyl ether, which on demethylation gave nataloemodin. Through the courtesy of Dr T. E. Wallis, Curator of The Museum of the Pharmaceutical Society, London, we received a specimen of nataloemodin monomethyl ether made by Léger. We demethylated this substance with hydrobromic acid in glacial acetic acid solution. The resulting nataloemodin, m.p. 216°, has properties quite different from 145-trihydroxy-2-methylanthraquinone, and is

clearly not identical with it. Funiculosin ($C_{15}H_{10}O_5$, m.p. 218°, triacetate, m.p. 205°, methoxy, nil) was isolated by Igaraci (1939) from laboratory cultures of *P funiculosum* Thom. Its molecular constitution was not established, though Igaraci expressed the opinion that it is either a trihydroxymethylanthraquinone or a dihydroxyhydroxymethylanthraquinone. This view is, however, difficult to reconcile with Igaraci's finding that funiculosin gives anthracene and not, as would be expected, a methylanthracene on zinc dust distillation. The identity of the anthracene was established by melting point, mixed melting point and analysis, and confirmed by conversion into anthraquinone. However, since *P islandicum* Sopp and *P funiculosum* Thom are regarded by Thom (1930) as closely related species, both being included in the *P funiculosum* series of the Biverticillata symmetrica, we prefer to regard the question of the identity of funiculosin with the *P islandicum* colouring matter as an open one for the present. For this reason we prefer in the meantime not to give the *P islandicum* colouring matter a trivial name.

EXPERIMENTAL

Cultures

Six different strains of *P islandicum* Sopp have been examined in the course of this work. They were all received, from the Thom-Raper collection, from Dr Kenneth B. Raper, Northern Regional Research Laboratory (N. R. R. L.), U.S. Department of Agriculture, Peoria, Illinois, U.S.A. Their history, kindly supplied by Dr Raper, is as follows.

1 Strain N. R. R. L. 1036 received originally in 1922 from F. M. Putterill, Cape Town, South Africa.

2 Strain N. R. R. L. 1037 received originally in 1924 from Dr Nakata. Isolated in Japan among moulds commonly found in the citrus industry.

3 Strain N. R. R. L. 1038 received originally in 1927 from Dr W. Schwartz from Germany.

4 Strain N. R. R. L. 1175 received originally in 1940 from Dr G. A. Ledingham, Ottawa, Canada.

5 Strain N. R. R. L. 1177 received originally in 1940 from Dr G. A. Ledingham, Ottawa, Canada. This strain was isolated from a sample of wet flour.

6 Strain N. R. R. L. 2115 received originally in 1942 from Dr Franz Lozet, Leopoldville, Belgian Congo.

Strain N. R. R. L. 1036 was received by us in October 1943, the other strains in December 1947.

145-Trihydroxy-2-methylanthraquinone was isolated from all six strains with the exception of Strain N. R. R. L. 1175 which, although morphologically a typical strain, appears to be biochemically atypical. Strain N. R. R. L. 1036 was used for the bulk preparation of 145-trihydroxy-2-methylanthraquinone.

Cultural characteristics of, and pigment formation by, different strains of *Penicillium islandicum*

Quantities of 350 ml. of Czapek Dox solution (glucose, 50.0 g, $NaNO_3$, 2.0 g, KH_2PO_4 , 1.0 g, KCl, 0.5 g, $MgSO_4 \cdot 7H_2O$, 0.5 g, $FeSO_4 \cdot 7H_2O$, 0.01 g, distilled water,

11) were distributed in each of eighteen 11 conical flasks, plugged with cotton wool and sterilized. A batch of three flasks was inoculated with one of each of the six strains of *P. islandicum* N R R L 1036, 1037, 1038, 1175, 1177 and 2115. One flask of each strain was harvested after 7, 14 and 21 days' incubation at 24° in the dark. The mycelium was separated by filtration, washed with water, and dried *in vacuo*.

Examination of the culture filtrates

The culture filtrates of all strains except 1175 were similar in appearance. They were all orange red in colour but varied somewhat in intensity, and filtered somewhat slowly. There remained on the filter paper a reddish deposit varying in amount with the different strains, and containing many microcrystalline orange red clusters of needles. On the other hand, strain 1175 gave an orange yellow culture filtrate with no sign of red colouring matter. The pH (Table 1, col. 3) varied within narrow limits for all strains.

Antibiotic activity The antibiotic activity (Table 1, col. 4) was measured by the serial dilution method against *Staphylococcus aureus*, Oxford H strain. All strains except 1175 at some period of growth totally inhibited the growth of *Staph. aureus* at dilutions of 1/20 to 1/80.

were washed with ethanol and finally with ether, and dried. The following weights were obtained: Strain 1036, 0.36 g, 1037, 0.58 g, 1038, 0.41 g, 1175, 0.17 g, 1177, 0.30 g, 2115, 0.76 g. The nature of this material has not yet been fully investigated, but it appears to be a phosphorylated polysaccharide.

Bromine reaction Addition of saturated bromine water (2 ml) to 10 ml of culture filtrate gave a characteristic reaction with all strains and at all stages of growth. The mixture remained clear for some time, but on standing overnight there was formed a heavy, yellow to orange, gelatinous precipitate.

Examination of mycelia

The appearance of the mycelia of strains 1036, 1037, 1038, 1177 and 2115 varied slightly, particularly in the early stages of growth, but in the later stages the strains were almost indistinguishable from each other. They all developed reddish drops on the surface of the mycelium which, at the end of the incubation period, consisted mainly of reddish brown to brownish red growth with small areas of green growth. The reverse of the mycelium was in all cases a dark reddish brown to brownish red. The colour of the mycelium of strain 1175 was much lighter, being yellow to orange with green areas and with a yellow to brown reverse.

Table 1 *Cultural characteristics of six strains of Penicillium islandicum Sopp, and yields of colouring matters formed by them*

Catalogue no (N R R L)	Incubation period (days)	pH after incubation	Antibiotic activity	Wt of mycelium (g)	Mycelium total wt (g)	'Fat' (g)	Na ₂ CO ₃ soluble pigment (g)	Na ₂ CO ₃ insoluble pigment (g)	Crystalline 1,4,5 tri- hydroxy 2 methyl anthra- quinone (g)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1036	7	3.6	1/20	2.13	10.34	0.87	1.41	1.19	0.20
	14	3.8	Nil	3.76					
	21	4.3	Nil	4.45					
1037	7	3.6	1/80	1.99	9.84	0.76	0.60	0.65	0.18
	14	3.8	1/80	3.96					
	21	4.2	1/20	3.89					
1038	7	3.6	1/20	1.56	7.49	0.41	0.31	0.42	0.04
	14	3.9	1/20	2.91					
	21	4.4	1/40	3.02					
1175	7	3.7	Nil	2.33	9.90	0.50	0.77	0.57	Nil
	14	3.7	Nil	3.62					
	21	4.0	Nil	3.95					
1177	7	3.6	1/20	1.76	9.46	1.06	0.82	1.16	0.09
	14	4.1	1/20	3.55					
	21	4.4	Nil	4.15					
2115	7	3.6	1/80	1.82	8.46	0.69	0.47	0.49	0.05
	14	3.7	1/40	3.26					
	21	3.8	1/40	3.38					

FeCl₃ reaction Addition of aqueous N/6 FeCl₃ to the culture filtrate gave with all strains a heavy, yellow to brown, amorphous precipitate, which usually appeared after 15 min and always appeared on standing overnight. To 300 ml of culture filtrate from each strain after 7 days' growth were added 30 ml of aqueous N/6 FeCl₃. The resulting precipitates were separated by centrifuging, washed with water, and dissolved in 2N HCl (8 ml), giving yellow to brownish red solutions. Addition of ethanol (50 ml) gave white to very pale yellow amorphous precipitates which

Small portions of the mycelia were pressed between layers of filter paper, and then part was immersed in cold conc H₂SO₄, and part in aqueous N NaOH. The mycelia of strains 1036, 1037, 1177 and 2115 developed deep to very deep violet colours with conc H₂SO₄ at all stages of growth. Strain 1038 gave a brownish red colour at 7 days, becoming deep reddish violet after 14 days, and deep violet after 21 days. With strain 1175 the corresponding colours were pale brown, dark reddish violet, and brownish red. With N NaOH, strains 1036, 1037, 1177 and 2115 developed a

series of green shades of colour, varying from clear emerald-green through olive green to bluish green. The colours with 1038 were shades of violet, and with 1175 reddish violet to a clear purple red.

The dried mycelia, after 7, 14 and 21 days' growth of each strain, were combined and powdered (Weights, see Table 1, col 6). The powder was extracted exhaustively with chloroform in a Soxhlet apparatus. A dark red extract was obtained in all cases except strain 1175 which gave a brown yellow extract. The solvent was removed by distillation *in vacuo* and the residual solid was extracted with light petroleum. The pale coloured light petroleum extract, on evaporation to dryness, left a red coloured 'fat' (Weights, see Table 1, col 7).

The material insoluble in light petroleum was dissolved in chloroform (11) and extracted with aqueous $N Na_2CO_3$. After centrifugal separation the Na_2CO_3 extracts were acidified. The resulting red precipitate was filtered, washed, and dried (Weights, see Table 1, col 8).

The residual chloroform solutions were evaporated to low bulk. In the cases of strains 1036 and 1037 crystals of crude 1 4 5 trihydroxy 2-methylanthraquinone (weights, 0.15 g, m.p. 214–215°, and 0.15 g, m.p. 212–215°, respectively) separated. With the other four strains no crystals separated. The mother liquors from 1036 and 1037 and the solutions from 1038, 1175, 1177 and 2115 were dried (total weight of Na_2CO_3 insoluble colouring matter, Table 1, col 9). The residues, except strain 1175, were sublimed in a high vacuum. A red sublimate was obtained in each case at 150–200°. The sublimate was crystallized once from glacial acetic acid, and the total weights of crystalline material isolated are given in Table 1, col 10. The different crystalline preparations all melted within the range 216–217.5°, and none of them depressed the m.p. of 1 4 5 trihydroxy 2-methylanthraquinone on admixture with a pure specimen of it, m.p. 218°.

Preparation of 1 4 5 trihydroxy-2-methylanthraquinone from Penicillium islandicum Sopp, strain N R R L 1036

Czapek Dox 5% glucose solution was distributed, in 350 ml. quantities, into a number of 11 conical flasks, plugged with cotton wool and sterilized by steaming for 0.5 hr. on each of 3 consecutive days. In typical preparations 50 such flasks were inoculated with a spore suspension of *P. islandicum*, strain N R R L 1036, and incubated in the dark at 24° for 19–21 days. At the end of this period the dark red mycelial mat was separated by filtration, washed with water, dried *in vacuo*, and ground in a coffee mill, yielding 201 g. of a brownish red powder. Treatment of the culture filtrate, which contained 0.1% of residual glucose, will form the subject of a future communication. The ground mycelium was freed from fat by boiling under reflux with three separate lots of 1 l. of light petroleum (b.p. 40–60°). The light orange coloured combined extracts were thoroughly extracted with aqueous 0.5N NaOH which, on acidification, gave 0.76 g. of fat contaminated colouring matter. The colourless residual light petroleum yielded 10.7 g. of 'fat' on removal of the solvent.

The colouring matters were extracted from the fat free powdered mycelium by boiling under reflux for 1–2 hr. each time with seven successive lots of 1 l. of chloroform. The chloroform solutions were all brownish red to orange red in

colour. A rough fractionation of the mixture of colouring matters was effected by thorough extraction of their chloroform solutions with aqueous Na_2CO_3 in which 1 4 5 trihydroxy 2-methylanthraquinone is not soluble. $N Na_2CO_3$ was used for the earlier and more concentrated chloroform solutions and 0.25N for the later ones. The somewhat emulsified mixtures were separated by centrifuging and the Na_2CO_3 extracts, varying in colour from brown with the earlier extracts to pure green with the later ones, were quickly acidified with HCl. The resulting dark red precipitates were separated by filtration, thoroughly washed with water, and dried. A dark red powder, total weight 19.15 g. was thus obtained and the fractionation of this material is at present under investigation.

Isolation of 1 4 5 trihydroxy 2-methylanthraquinone from the chloroform solutions, free from Na_2CO_3 soluble substances, may be accomplished by either of the following methods. (a) The chloroform solutions were washed with water, filtered and evaporated to crystallization point. Second crops were obtained by further evaporation and crystallization. A total yield of 6.19 g. of crude 1 4 5 trihydroxy 2-methylanthraquinone was obtained. The crude product was purified by repeated recrystallization from chloroform or glacial acetic acid yielding shining red plates, m.p. 218°. On removal of the solvent from the original chloroform mother liquors there remained 18.82 g. of a mixture of colouring matters. (b) The chloroform solutions were combined and thoroughly extracted with aqueous 0.5N NaOH. This treatment removed the remainder of the colouring matters leaving an almost colourless chloroform solution. Acidification of the bluish purple NaOH extract with HCl yielded a red amorphous solid which was separated by filtration, washed with water, and dried. This was heated in small portions in a high vacuum when 1 4 5-trihydroxy 2-methylanthraquinone sublimed readily at 150–170°. The combined sublimate, after one crystallization from chloroform, yielded pure 1 4 5 trihydroxy 2-methylanthraquinone, m.p. 218°, weight 6.1 g. Although this method is somewhat tedious it readily gives a pure product.

The Na_2CO_3 extracted chloroform solutions contain, in addition to 1 4 5 trihydroxy 2-methylanthraquinone, other NaOH soluble colouring matters, which are left in the chloroform mother liquors if method (a) is used, or remain unextracted below 200° if method (b) is used. The nature of these colouring matters is being investigated.

The total content of crude mixed colouring matters in the dried mycelium of *P. islandicum*, strain N R R L 1036, is rather remarkable. In a number of different preparations the average yield obtained was about 20% of the weight of the dried mycelium.

General properties of 1 4 5-trihydroxy-2-methylanthraquinone Derivatives

1 4 5 Trihydroxy 2-methylanthraquinone crystallizes from chloroform or glacial acetic acid in large, dark red, lustrous plates or leaflets, m.p. 218°. (Found C, 66.8, 66.9, H, 4.0, 3.9, C CH_3 , 6.0%, CH_3O , nil. $C_{16}H_{10}O_5$ requires C, 66.7 H, 3.7, C CH_3 , 5.6%.) It dissolves fairly readily in chloroform or acetone, is rather less soluble in glacial acetic acid, ethanol and ether, and is almost insoluble in light petroleum. It is insoluble in $N NaHCO_3$, $N Na_2CO_3$ and $N NH_4OH$, but dissolves readily in $N NaOH$ to a violet solution, the colour of which fades to a pale yellow after a few hours' exposure.

to air. Its solution in cold conc H_2SO_4 is bright purple red in bulk and bluish red in thin layers with a fiery red fluorescence. Its solution in glacial acetic acid is yellow orange in colour with a green fluorescence. It readily sublimed without decomposition in a high vacuum at 160° .

1 4 5 Triacetoxy 2 methylanthraquinone A solution of crude 1 4 5 trihydroxy-2 methylanthraquinone (3 36 g) in 35 ml of acetic anhydride containing 2% (v/v) conc H_2SO_4 was boiled for 2 min. The cooled brown solution was poured into iced water (1 l) with stirring. The resulting yellow solid was crystallized twice from ethanol and 4 times from glacial acetic acid to give 1 4 5 triacetoxy 2 methylanthraquinone as pale yellow needles with a constant m p of 208° (Found C, 63 7, 63 7, H, 4 2, 4 0% $\text{C}_{21}\text{H}_{16}\text{O}_8$ requires C, 63 6, H, 4 1%)

1 4 5 Triacetoxy 2 methylanthraquinone (0 5 g) was heated with aqueous 2N NaOH (30 ml) on a boiling water bath in an atmosphere of N_2 . The acetate dissolved in 10 min to an intensely violet solution. Heating was continued for a further hour when 2N HCl (35 ml) was added. The resulting red amorphous precipitate (0 32 g) was separated by filtration, washed well with water, dried and crystallized from chloroform. 1 4 5 Trihydroxy 2 methylanthraquinone, weight 0 16 g, m p 218° , was thus obtained as dark red plates.

1 4 5-Trimethoxy 2-methylanthraquinone 1 4 5-Trihydroxy-2 methylanthraquinone (0 25 g) was dissolved in boiling anhydrous acetone (25 ml), and redistilled dimethyl sulphate (1 25 ml) and anhydrous K_2CO_3 (1 25 g) were added in 0 25 ml and 0 25 g portions at suitable intervals during 8 hr boiling under reflux. The resulting yellow solution was separated by filtration from the mineral salts, which were thoroughly extracted with hot acetone. Evaporation of the solvent *in vacuo* and crystallization of the residue from ethanol gave 1 4 5 trimethoxy 2 methylanthraquinone (0 20 g) as orange yellow needles, m p 161° , unchanged on further crystallization and sublimation at 150° in a high vacuum (Found C, 69 3, 69 2, H, 5 4, 5 4, CH_3O , 27 2, 27 7%, mol.wt (cryoscopic in camphor) 320, 314 $\text{C}_{15}\text{H}_{10}\text{O}_5$ requires C, 69 2, H, 5 2, $3\text{CH}_3\text{O}$, 29 8%, mol.wt 312). In contrast to the parent substance 1 4 5 trimethoxy 2 methylanthraquinone is insoluble in N NaOH. It dissolves in cold conc H_2SO_4 to a blue solution with a faint purple fluorescence, and in glacial acetic acid to a pale yellow non fluorescent solution.

Oxidation of 1 4 5 trihydroxy-2-methylanthraquinone Formation of cynodontin (V)

Finely powdered MnO_2 (0 75 g) was added in portions over a period of 5 min and with mechanical stirring to a solution of 1 4 5 trihydroxy 2 methylanthraquinone (1 g) in conc H_2SO_4 (7 5 ml). The temperature was maintained at 80° and stirring was continued for a further 0 5 hr. The mixture was cooled, poured into water (500 ml) and then boiled for 10 min. The original brown violet precipitate became redder on heating. After cooling, the red solid was separated by filtration, washed and dried. The dried material was extracted exhaustively with chloroform in a Soxhlet apparatus, collecting four fractions which were evaporated and crystallized separately giving fraction I, 0 15 g, m p $238-240^\circ$, II, 0 18 g, m p $246-247^\circ$, III, 0 23 g, m p $247-249^\circ$, IV, 0 10 g, m p $252-253^\circ$, V (from the combined mother liquors), 0 14 g, m p $219-224^\circ$.

Fractions I, II, III and IV were combined and purified by acetylation in the usual way with acetic anhydride and conc H_2SO_4 . The resulting acetate was crystallized twice from ethanol yielding 0 40 g of fine yellow needles, m p $228-229^\circ$, which did not depress the m p of synthetic tetraacetylcynodontin (Anslow & Raistrick, 1940b) similarly crystallized (Found C, 60 45, 60 8, H, 4 0, 4 0% Calc for $\text{C}_{23}\text{H}_{18}\text{O}_{10}$ C, 60 8, H, 4 0%).

The acetate (0 32 g) was hydrolyzed with aqueous 2N NaOH (20 ml) on a boiling water bath in an atmosphere of N_2 . The resulting deep blue solution, on acidification, yielded 0 19 g of regenerated colouring matter which was crystallized, first from pyridine (20 ml, with charcoal), and then from glacial acetic acid (35 ml). There was thus obtained 0 11 g of brownish leaflets with a magnificent bronze lustre which melted at $259.5-260.5^\circ$, alone or in admixture with natural cynodontin, m p $259.5-260.5^\circ$, prepared from *Helminthosporium cynodontis* Marignoni (Found C, 63 3, 63 0, H, 3 6, 3 6% Calc for $\text{C}_{15}\text{H}_{10}\text{O}_4$ C, 62 9, H, 3 5%). The two specimens also gave identical reactions in the following tests: N Na_2CO_3 , insoluble, N NaOH, dissolved to a deep blue violet solution, cold conc H_2SO_4 , dissolved to a blue solution with a fine red fluorescence, glacial acetic acid, bluish red solution with a weak greenish yellow fluorescence.

Reduction of 1 4 5-trihydroxy-2-methylanthraquinone Formation of chrysophanic acid (VI)

A solution of 1 4 5 trihydroxy 2 methylanthraquinone (1 g) in glacial acetic acid (20 ml) was boiled for 5 hr under reflux with red phosphorus (1 g) and HI (4 ml, sp gr 1 7). The mixture was cooled slowly, and the crystals that formed were separated by filtration. When recrystallized from glacial acetic acid (50 ml, with charcoal) there was obtained 0 48 g of light orange red plates which had all the colour reactions of chrysophanic acid anthranol and melted at $200-204^\circ$ (lit $205-210^\circ$).

The anthranol (0 25 g) was dissolved in hot glacial acetic acid (10 ml). A solution (1 4 ml) of CrO_3 (10%, w/v) in glacial acetic acid was added with shaking and the mixture was maintained at 60° . A further 1 4 ml of CrO_3 solution were added in portions at intervals, and, after a total oxidation period of 35 min at 60° , the mixture was cooled. Yellow orange crystals (0 13 g, m p $191-193^\circ$) separated, and this material was purified by sublimation in a high vacuum at $140-160^\circ$. The orange yellow sublimate was dissolved in chloroform, extracted three times with 10 ml of 0 5N aqueous Na_2CO_3 , and then washed with water. After removal of the chloroform, the resulting solid was crystallized from ethanol, yielding glistening orange yellow plates (0 06 g, m p 194°) (Found C, 70 6, 70 8, H, 4 2, 4 3% Calc for $\text{C}_{15}\text{H}_{10}\text{O}_4$ C, 70 9, H, 4 0%). The substance proved to be chrysophanic acid, a pure specimen of which was prepared from a sample of Kahlbaum's chrysophanic acid kindly given to us by Dr H D Springall, Department of Chemistry, University of Manchester. This authentic specimen melted at 195° and a mixture of the two specimens melted at 195° . The two specimens also gave identical reactions in the following tests: N Na_2CO_3 , insoluble, N NaOH, dissolved to a red solution, changing slowly to orange red with the formation of a red precipitate, cold conc H_2SO_4 , dissolved to a red non fluorescent solution, glacial acetic acid, dissolved to a yellow solution.

The reduction product, chrysophanic acid (0.13 g), was acetylated in the usual way with acetic anhydride (2 ml) containing 1 drop of conc H_2SO_4 . The solid that separated on adding ice was crystallized from ethanol (with charcoal) and gave yellow leaflets, m p $207-208^\circ$ (Found C, 67.1, H, 4.3%. Calc for $\text{C}_{16}\text{H}_{14}\text{O}_6$ C, 67.45, H, 4.2%). It was compared with an authentic specimen of diacetyl chrysophanic acid, kindly supplied by the Wellcome Research Institution and prepared originally by Dr F B Power. This specimen melted at $208-209^\circ$, and a mixture of the two at $208-209^\circ$.

Comparisons of 1 4 5 trihydroxy-2-methylantraquinone

(a) *With nataloemodin* A specimen of methylnataloemodin (nataloemodin monomethyl ether) prepared originally by Léger (1902, 1905) was kindly given to us by Dr T E Wallis, Curator of the Museum of the Pharmaceutical Society, London. A solution of methylnataloemodin (54 mg) in 5 ml of equal volumes of glacial acetic acid and constant b p aqueous HBr (46-48%) was boiled under reflux for 6 hr. The initially yellow solution slowly became brown and on standing overnight deposited nataloemodin in orange brown needles in almost theoretical yield. These were recrystallized from acetic acid (80% v/v, 16 ml) and yielded pure nataloemodin (0.04 g) as fine golden yellow needles, m p 216° . A mixture of nataloemodin with 1 4 5 trihydroxy 2 methylantraquinone, m p 218° , melted at $185-192^\circ$. Nataloemodin gave the following colour reactions: $\text{N Na}_2\text{CO}_3$, readily dissolves to a cerise solution, 0.5N NaOH, violet solution not fading quickly, cold conc H_2SO_4 , red-violet solution almost indistinguishable from aqueous KMnO_4 , glacial acetic acid, yellow non fluorescent solution. The two substances are clearly quite different compounds.

(b) *With helminthosporin (III)* A mixture of 1 4 5 trihydroxy 2 methylantraquinone, m p 218° , with hel-

minthosporin, m p $225-226^\circ$, from *Helminthosporium gramineum* Rabenhorst melted at $192-200^\circ$, a mixture of 1 4 5 triacetoxy 2 methylantraquinone, m p 208° , with triacetyl helminthosporin, m p $223-225^\circ$, melted at $199-203^\circ$. It is thus clear that the two substances are quite different compounds, although in comparative colour tests with N NaOH, cold conc H_2SO_4 and glacial acetic acid the colours given by the two substances were almost indistinguishable.

SUMMARY

1 The dried mycelium of laboratory cultures of *Penicillium islandicum* Sopp has been shown to contain about 20% of its weight of a complex mixture of colouring matters.

2 One constituent of this mixture has been isolated in a pure form from five different strains of *P. islandicum* and has been shown to be the hitherto undescribed 1 4 5-trihydroxy-2-methylantraquinone.

3 1 4 5-Trihydroxy-2-methylantraquinone crystallizes in lustrous dark red plates, m p 218° , and forms a triacetate, pale yellow needles, m p 208° , and a trimethyl ether, orange yellow needles, m p 161° .

4 On oxidation with manganese dioxide and concentrated sulphuric acid, 1 4 5-trihydroxy-2-methylantraquinone yields cynodontin, on reduction with concentrated hydriodic acid and red phosphorus and oxidation of the resulting anthranol with chromic acid, chrysophanic acid is formed.

5 The close structural relationship existing between a number of mould colouring matters is discussed.

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Studies in Detoxication

21 THE FATES OF QUINOL AND RESORCINOL IN THE RABBIT IN RELATION TO THE METABOLISM OF BENZENE

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The study of the metabolic fates of quinol and resorcinol was undertaken to elucidate certain aspects of the metabolism of benzene and phenol (see Porteous & Williams, 1949*a, b*). We have already reported on the fate of catechol (Garton & Williams, 1948), and a further study of the fate of phenol is in progress. Quinol is also of importance in industry as a photographic chemical and as a food preservative. Its position as an industrial hazard has been discussed recently by Sterner, Oglesby & Anderson (1947).

There is little exact information concerning the fate of these two phenols in the body. Baumann & Preusse (1879) found that a dog given 0.5 g quinol excreted a quinol ethereal sulphate though no free quinol could be detected. von Mering (1876) also demonstrated the presence of a quinol ethereal sulphate in the urine of rabbits fed with arbutin (quinol monoglucoside). Kulz (1890) found that quinol fed rabbits excreted a glucuronide. Baumann (1878-9) reported that resorcinol raised the ethereal

sulphate output in dogs, and Kulz (1890) showed that it formed a glucuronide in rabbits and hares.

EXPERIMENTAL

Quantitative experiments

The rabbits (giant chinchilla) used were kept on a diet of Lever's cubes (50 g/day) and water *ad lib*. Quinol (m.p. 169°) and resorcinol (m.p. 110°), dissolved in water, were fed by stomach tube. Doses up to 0.4 g/kg of quinol and 0.5 g/kg of resorcinol could be administered without apparent toxic effect. Doses of 0.45 g/kg quinol and 0.6 g/kg resorcinol caused temporary muscular twitching and an increased rate of respiration. In most of the present experiments doses of 0.1-0.2 g/kg were used.

Glucuronic acid and ethereal sulphate excretions were determined as described in earlier papers in this series (Hanson, Mills & Williams, 1944; Williams, 1938). The results, given in Table 1, show that with quinol 30% of the dose is excreted as an ethereal sulphate and 43% as a monoglucuronide, and with resorcinol 13.5% is excreted as a sulphate and 52% as a monoglucuronide.

Table 1. *The excretion of glucuronic acid and ethereal sulphate by rabbits receiving quinol or resorcinol orally*

(The dose of dihydroxybenzene was 100 mg/kg of body wt.)

Rabbit no	Wt (kg)	Dose (mg)	Average normal values		'Extra' excretion		Percentage of dose excreted		
			Ethereal sulphate (mg SO ₃ / day)	Glucuronic acid (mg /day)	Ethereal sulphate (mg SO ₃ / day)	Glucuronic acid (mg /day)	As mono sulphate	As mono glucuronide	Total conjugation
Quinol fed									
122	3 15	630*	25 1	—	129 4	—	28 3	—	—
123	3 50	700*	14 7	—	200 3	—	39 3	—	—
124	3 05	710†	13 9	—	126 8	—	24 6	—	—
98	3 15	315	17 1	168	86 7	232	37 8	41 8	79 6
90	2 95	295	—	116	—	184	—	35 4	—
81	2 85	285	—	150	—	213	—	42 4	—
124	2 90	290	16 7	126	56 2	250	26 6	48 9	75 5
125	3 10	310	18 3	155	56 2	231	24 9	42 2	67 1
126	3 05	305	19 2	156	62 6	245	28 3	46 6	74 8
Resorcinol fed									
124	2 85	285	13 8	106	27 8	272	13 4	54 1	67 5
125	3 25	325	15 8	105	31 2	295	13 2	51 5	64 7
126	3 10	310	15 2	107	39 7	277	14 0	50 6	64 6

* Dose 200 mg/kg † Dose 230 mg/kg

*Isolation of metabolites**Metabolites of quinol*

Quinol urine is normal in colour when freshly voided, but darkens considerably on standing. The slightly alkaline urine gives a dark green brown colour with 2% FeCl_3 . It reduces very slightly to Fehling's and Benedict's reagents and gives an intense Tollens test for glucuronic acid.

Isolation of free quinol The 24 hr urine (340 ml), collected after feeding a total of 4 g quinol to three rabbits, was filtered, treated with 0.1 g $\text{Na}_2\text{S}_2\text{O}_4$ and extracted continuously for 6 hr with peroxide free ether. The extract was evaporated at 30° to a clear pale yellow gum (90 mg). The gum was dissolved in 5 ml 2.5N NaOH and treated with 0.2 g *p*-toluenesulphonyl chloride in 5 ml acetone. After it had been shaken for 10 min the mixture was poured into 50 ml water. The crystals (10 mg, 0.065% of dose) which formed were collected and dried and identified as quinol di-*p*-toluenesulphonate, m.p. 155° alone and 157° mixed with an authentic specimen, m.p. 159° . A separate experiment showed that quinol added to normal urine could be recovered quantitatively by ether extraction after the addition of $\text{Na}_2\text{S}_2\text{O}_4$.

The glucuronide of quinol **Isolation of the quinol glucuronide gum** A 24 hr urine (945 ml) was collected after feeding a total of 12 g quinol to twelve rabbits and the basic lead acetate precipitate prepared in the usual manner. The precipitate was well washed with water and Pb removed with H_2S . The Pb free filtrate was aerated, treated with charcoal, filtered and evaporated to a gum (18 g) *in vacuo* at $50-60^\circ$. The gum was dissolved in 10 ml absolute ethanol and kept at 0° overnight. A precipitate (0.5 g) of inorganic material was removed, and the solution was then evaporated to dryness. The gum, presumably quinol glucuronide, was purified by dissolving in ethanol, filtering and evaporating several times and yielded 16.5 g of a clear brown gum. It was acid to litmus, gave a pale green colour with FeCl_3 which deepened on making slightly alkaline with NaHCO_3 and an intense Tollens naphthoresorcinol test. It was insoluble in ether, benzene and light petroleum.

Quinol monoglucuronide methyl ester The gum (3 g) was dissolved in 50 ml ethanol and 200 ml of a saturated solution of diazomethane in ether added. The mixture was kept overnight, filtered from a little sludge and evaporated to dryness. The treatment with diazomethane was repeated twice. Finally a clear yellow, neutral, ether soluble gum (2.47 g) was obtained which could not be induced to crystallize. The *p*-hydroxyphenylglucuronide methyl ester gave a blue colour with FeCl_3 , indicating the presence of a free phenolic OH group (Found OCH_3 , 10.2; $\text{C}_{13}\text{H}_{16}\text{O}_8$ requires OCH_3 , 10.3%).

Acetylation of quinol glucuronide methyl ester The above methyl ester (2.47 g) was dissolved in 10 ml pyridine and 15 ml acetic anhydride. After it had been kept overnight at room temperature the mixture was poured into 100 ml of ice water. After the mixture had been stirred for some time at 0° crystallization occurred. The crystals (3.25 g, m.p. 148°) were collected and recrystallized from ethanol. The *p*-acetoxyphenyl triacetyl β -glucuronide methyl ester formed long, colourless needles, m.p. 151° , $[\alpha]_D^{16} -21.8^\circ$ (c, 1 in acetone) (Found C, 53.7, H, 5.1, OCH_3 , 6.9; $\text{C}_{21}\text{H}_{24}\text{O}_{12}$ requires C, 53.85, H, 5.2, OCH_3 , 6.6%). The compound was insoluble in water, sparingly soluble in ethanol, soluble

in ether and readily soluble in acetone. It gave the naphthoresorcinol reaction on prolonged heating (5 min) with the reagents.

Hydrolysis of acetylated quinol monoglucuronide methyl ester *p*-Acetoxyphenyl triacetylglucuronide methyl ester (1.5 g) in 15 ml ethanol and 100 ml 4N HCl was heated under reflux for 4 hr on the water bath. After cooling, the brown solution was extracted with 5×50 ml peroxide free ether. The extracts were evaporated to dryness *in vacuo* and the partially crystalline residue dissolved in 10 ml 2.5N NaOH and treated with 0.5 g *p*-toluenesulphonyl chloride. After shaking for 20 min the mixture was poured into 150 ml cold water. The crystalline precipitate (315 mg) of quinol di-*p*-toluenesulphonate was recrystallized from absolute ethanol to give colourless prisms, m.p. and mixed m.p. 159° .

The ethereal sulphate fraction of quinol urine If it is assumed that quinol forms a monosulphate, then, according to Table 1, the proportion of quinol conjugated with sulphuric acid is twice that of catechol (Garton & Williams, 1948) or resorcinol. It was, therefore, necessary to show whether quinol monosulphuric acid or quinol disulphuric acid is excreted by rabbits.

Attempts to isolate the ethereal sulphate and compare it with synthetic material (see below) were unsuccessful, but we proved it to be a monosulphate by the following procedure. The urine (1025 ml), collected after feeding a total of 10 g quinol to ten rabbits, was treated with 0.5 g K_2CO_3 and reduced *in vacuo* at 40° to 250 ml. The resulting dark brown liquid was clarified with 0.25 g $\text{Na}_2\text{S}_2\text{O}_4$ and saturated with $(\text{NH}_4)_2\text{SO}_4$, 250 ml acetone were added and the mixture filtered at the pump, the precipitated $(\text{NH}_4)_2\text{SO}_4$ being washed with 150 ml acetone. The combined filtrates were transferred to a separating funnel and the aqueous layer removed. The acetone solution was treated with 0.5 g K_2CO_3 and concentrated *in vacuo* at 40° to 200 ml. This was now poured into 2 l dry acetone. A dark brown viscous layer containing glucuronides separated and was discarded. The clear golden acetone layer was again reduced *in vacuo* at 40° to 150 ml. Glucuronides were still present and so the solution was poured into 1 l dry acetone and the brown gum of glucuronide again removed. The acetone solution was again evaporated at 40° to a gummy crystalline mass. The product was practically free from glucuronides, and contained no inorganic sulphate. The crystals in it were identified as urea. This material contained the ethereal sulphates of quinol urine, but these could not be isolated. It was, therefore, dissolved in 100 ml 50% aqueous ethanol, and its content of free and total quinol and ethereal sulphate estimated as follows.

Quinol was estimated by the iodometric method of Wieland (1910), as described by Neuberger (1947) for the determination of homogentisic acid in urine. Urea does not interfere with the determination. The above ethereal sulphate fraction contained 55 mg of free quinol. For the estimation of total quinol, 5 ml of the ethereal sulphate fraction were boiled under reflux with 10 ml of 0.3N-HCl for 30 min, cooled and the quinol estimated iodometrically. The combined quinol found was 1.045 g.

A gravimetric determination of ethereal sulphate showed that the fraction contained 0.8561 g SO_3 . Thus the ratio ethereal sulphate SO_3 /combined quinol in the fraction is $0.8561/1.045 = 0.82$. For quinol monosulphuric acid the calculated ratio is 0.73, whereas for quinol disulphuric acid

it is 1.45. These results indicate that the ethereal sulphate of quinol urine is quinol monosulphuric acid.

Search for quinol oxidation products In an earlier paper (Garton & Williams, 1948) we showed that, in the rabbit, hydroxyquinol is an oxidation product of catechol. Since catechol, quinol and hydroxyquinol are metabolites of benzene (Porteous & Williams, 1949*b*), it is important to know whether quinol is also oxidized to hydroxyquinol. A careful search was made for this phenol in the urine collected 1, 2 and 3 days after feeding a total of 4 g quinol to three rabbits. No trace of free or combined hydroxyquinol was found. The colour reactions used have been described in earlier papers (Garton & Williams, 1948; Porteous & Williams, 1949*b*).

Metabolites of resorcinol

Resorcinol urine is slightly darker than normal rabbit urine, but does not darken on standing as does quinol urine. The slightly alkaline urine is non-reducing, gives no colour with FeCl_3 , but gives a strong naphthoresorcinol reaction. It contains free resorcinol, giving a purplish red colour in the 2,6-dichloroquinone chloroimide test of Porteous & Williams (1949*b*).

Isolation of free resorcinol Resorcinol (1 g) was fed to each of six rabbits. The slightly alkaline 24 hr urine (625 ml) was filtered through glass wool and then extracted continuously for 8 hr with peroxide-free ether. Evaporation of the extract at 20° yielded a partially crystalline product (1.97 g) from which 0.5 g resorcinol (m.p. and mixed m.p. 110° , after crystallization from dry benzene) was obtained. The mother liquors, on benzylation, yielded 0.255 g of resorcinol dibenzoate (m.p. and mixed m.p. 117°). A further 8 hr extraction removed all the free resorcinol from the urine and 0.27 g of the dibenzoate was obtained. Thus a total of 11.4% of the resorcinol fed was recovered from the urine in the free state.

The glucuronide of resorcinol: isolation of the resorcinol glucuronide gum The glucuronide gum (22 g) was prepared by the usual basic lead acetate procedure from the 24 hr urine (1.5 l) of eight rabbits which had collectively received 12 g of resorcinol. The gum was purified by dissolution in absolute ethanol and filtering. In this way 23.5 g of a viscous brown gum were obtained consisting mainly of resorcinol monoglucuronide. This gum was acidic, non-reducing, gave an olive green colour with FeCl_3 and an intense naphthoresorcinol reaction. It was readily soluble in water, ethanol and ethyl acetate. It could not be crystallized, or induced to give crystalline salts with organic bases.

Since resorcinol monoglucuronide is a phenol in which the position para to the phenolic OH group is unsubstituted, it should give a coloration with 2,6-dichloroquinone chloroimide. In fact the gum gives an intense purple colour with this reagent in slightly alkaline solution (NaHCO_3) and at all values of pH up to 10. This reaction in itself shows that the glucuronide of resorcinol is a monoglucuronide. Resorcinol itself gives a purplish red colour with 2,6-dichloroquinone chloroimide. The quinol monoglucuronide gum which has already been described gives no colour with 2,6-dichloroquinone chloroimide, a result to be expected since quinol glucuronide is a *p*-substituted phenol.

Resorcinol monoglucuronide methyl ester The above gum (5 g) was methylated in ethanol with ethereal diazomethane. In *Hydroxyphenylglucuronide methyl ester* was

obtained as an ether-soluble, neutral, clear brown gum (Found OCH_3 , 11.4. $\text{C}_{13}\text{H}_{16}\text{O}_8$ requires OCH_3 , 10.3%), which gave a transient purple colour with FeCl_3 .

Acetylation of resorcinol glucuronide methyl ester The ester (3.6 g) was acetylated at room temperature with 10 ml pyridine and 15 ml acetic anhydride. On pouring the mixture into water a yellow oil separated. The oil was taken up in 50 ml CHCl_3 and the solution washed successively with 10% Na_2CO_3 , 2*N* HCl and water. After drying over anhydrous CaCl_2 , the chloroform was evaporated, leaving 4 g of a pale yellow gum which did not crystallize. The acetylation and extraction procedure was therefore repeated, but again a yellow gum was obtained. The gum was dissolved in 30 ml ethanol and water was added dropwise with stirring. After vigorous scratching the precipitated material crystallized and eventually 3.16 g of small colourless needles, m.p. 112° , were obtained. The *m*-acetoxyphenyl triacetyl β -glucuronide methyl ester was recrystallized from ethanol, forming small needles, m.p. 113 – 114° , $[\alpha]_D^{20}$ -24.5° (c, 2 in acetone). (Found C, 54.25, H, 5.2, OCH_3 , 6.9, CH_3CO , 39.8. $\text{C}_{21}\text{H}_{24}\text{O}_{12}$ requires C, 53.85, H, 5.2, OCH_3 , 6.6, CH_3CO , 36.8%). The compound was soluble in ethanol, acetone and chloroform, but insoluble in water. It gave a strong naphthoresorcinol reaction when boiled for 2–3 min with the reagents.

Hydrolysis of the acetylated resorcinol monoglucuronide methyl ester The ester (3 g) was refluxed on a water bath with 15 ml ethanol and 100 ml 4*N* HCl for 2.5 hr. A red-brown solution was formed which, on cooling, deposited a reddish flocculent precipitate. This precipitate is presumably the result of the condensation of the free resorcinol with the free glucuronic acid formed during hydrolysis of the ester (cf. the naphthoresorcinol reaction). The solution was extracted with 4×75 ml ether and the combined extracts dried over anhydrous Na_2SO_4 . Removal of the ether at 30° left a pale brown gum (1.53 g). On benzoylating the gum and pouring the product into water, an oil separated. This was dissolved in 30 ml acetone which was then poured into 100 ml water giving 350 mg of small colourless plates of resorcinol dibenzoate, which, after recrystallization from aqueous ethanol, had m.p. and mixed m.p. 117° with authentic material.

The residual solution, after removal of resorcinol by ether, contained glucuronic acid as indicated by the Tollens test and by the reduction of Benedict's and Fehling's solutions.

Search for resorcinol oxidation products Resorcinol urine was also carefully examined for hydroxyquinol, pyrogallol and phloroglucinol. No trace of any of these phenols was found in either the ethereal sulphate or glucuronide fraction of the urine collected 24 or 48 hr after feeding a total of 6 g resorcinol to six rabbits.

The colour tests used were those described by Porteous & Williams (1949*b*), together with a specific colour reaction for phloroglucinol. In this test traces of phloroglucinol give a red-orange colour with 0.25% aqueous quinol and 0.5*N* KOH (cf. Porteous & Williams, 1949*a*).

DISCUSSION

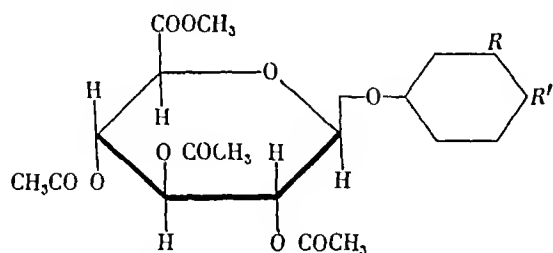
The quantitative aspects of the metabolic fates of the *o*-, *m*- and *p*-dihydroxybenzenes are summarized in Table 2, the values for catechol being quoted from Garton & Williams (1948). Quinol and catechol

Table 2 *The metabolism of the isomeric dihydroxybenzenes in the rabbit*

Compound	Conjugation (% of dose)			G/E	Other metabolites detected
	As ethereal sulphate (E)	As glucuronide (G)	Total		
Catechol	18	70	88	3.9	Free catechol (2% isolated), traces of hydroxyquinol as an ethereal sulphate
Resorcinol	13.5	52	65.5	3.9	Free resorcinol (11.4% isolated) (combined + free = 77%)
Quinol	30	43	73	1.4	Free quinol in traces (0.065% isolated)

are excreted almost entirely conjugated and although resorcinol is also highly conjugated, appreciable amounts (11–12% by isolation) are excreted in the free state. The ratio glucuronide/etheral sulphate is approximately 4 for both catechol and resorcinol, but only 1.4 for quinol, which is more highly conjugated with sulphate than its isomers. The proportion of the sulphate conjugation of quinol is twice that of its isomers and at first we suspected that quinol was being excreted as a disulphuric ester. The evidence presented, however, indicates that the ethereal sulphate is quinol monosulphuric acid. A consideration of the figures for the sulphate conjugation of resorcinol suggests that there is no reason to believe that resorcinol forms other than a monosulphuric ester.

The glucuronides of resorcinol and quinol have been shown to be monoconjugates by the isolation and characterization of the crystalline acetylated methyl esters (I) and (II) (*m*- and *p*-acetoxyphenyl-2,3,4-triacetyl- β -D-glucuronide methyl esters).

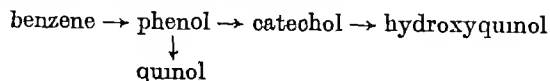


I ($R=\text{CH}_3\text{COO}$, $R'=\text{H}$) II ($R=\text{H}$, $R'=\text{CH}_3\text{COO}$)

Our proof of the structure of catechol monoglucuronide (Garton & Williams, 1948) depended on the fact that, on methylation with ethereal diazomethane, the glucuronide was converted to *o*-methoxyphenylglucuronide methyl ester. Hydrolysis of the crystalline triacetyl derivative of this ester yielded catechol monomethyl ether, thus proving that the original glucuronide contained one free phenolic hydroxyl group. In the present work, however, we found that when quinol and resorcinol glucuronides were methylated with diazomethane only the carboxyl groups were methylated. The non-crystalline methyl esters of quinol and resorcinol glucuronides obtained gave colours with ferric chloride, thus showing the

presence of a free phenolic hydroxyl group. The proof that these glucuronides were monoconjugates, therefore, depends on the elementary analysis of the crystalline acetylated methyl esters (I) and (II) and on colour tests for free phenolic hydroxyl groups. The purified non-crystalline quinol monoglucuronide gave a pale green colour with ferric chloride, its methyl ester a blue colour, whereas non-crystalline resorcinol monoglucuronide gave an olive-green and its methyl ester a transient purple colour. Further evidence that resorcinol glucuronide contains a free phenolic group was obtained by the use of 2,6-dichloroquinone chloroimide (see p. 236).

Neither quinol nor resorcinol undergoes further oxidation *in vivo* as does catechol. We searched very carefully for trihydric phenols, but we found no indication of their presence. In an earlier paper (Porteous & Williams, 1949b) it was shown that phenol, catechol, quinol and hydroxyquinol were oxidation products of benzene in the rabbit. The present paper shows that hydroxyquinol is not a metabolite of quinol, though we have shown it to be formed from catechol (Garton & Williams, 1948). Thus the hydroxyquinol in benzene urine must be derived from catechol. A detailed study of the metabolism of phenol in the rabbit (Garton & Williams, unpublished) has shown that phenol gives rise to catechol, quinol and hydroxyquinol. Porteous & Williams (1949b) suggested that benzene was oxidized in the rabbit as follows:



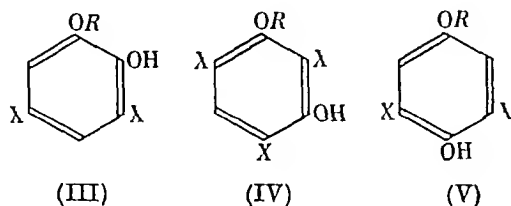
This scheme has now substantial experimental support.

The present work also raises the question, why is catechol oxidized to hydroxyquinol, whereas quinol and resorcinol are not? There are three possible answers to this question.

First, the enzymic systems which occur in animal tissues may oxidize only catechol. It is known, for example, that the tyrosinase system will oxidize catechol, but not quinol and resorcinol (Nelson & Dawson, 1944; Sumner & Somers, 1947). Nevertheless, Cadden & Dill (1942) have obtained from pig kidney a polyphenolase which oxidizes both

catechol and quinol This enzyme resembles the plant enzyme laccase which oxidizes quinol and catechol to quinones, but not resorcinol (Yakushiji, 1940) Samisch (1937) claims that lemon leaves contain a metaphenolase which oxidizes resorcinol very slowly

A second possible explanation may be derived from a consideration of the structures of the three phenols and their monoconjugates It is well known that phenols are oxidized either *o*- or *p*- to the existing —OH group In the formulae (III), (IV) and (V), the *o* and *p*-positions are marked with *X*



and it is to be noted that in the resorcinol (IV) and quinol (V) derivatives, the positions *X* are all ortho to an existing group There may, therefore, be steric hindrance to oxidation in these positions There is one position *X* in the catechol derivative (III) which is not subjected to steric hindrance by ortho substituents, and catechol is the only dihydric phenol which is metabolized to hydroxyquinol More information is required, however, on the metabolism of hydroxyquinol before the plausibility of

this explanation can be assessed Preliminary work on hydroxyquinol is in hand

A third explanation takes into account the time factor If quinol and resorcinol were excreted more rapidly than catechol, then it is possible that catechol, because of its longer stay in the body, would have more chance of being oxidized than the others We have no exact data on relative rates of excretion of these phenols, except that we observed that in the doses used in the present work all three were almost completely excreted within 24 hr of dosing

SUMMARY

1 The metabolism of quinol and resorcinol in the rabbit has been studied

2 Quinol is excreted almost entirely as mono glucuronide (43 %) and monosulphate (30 %) Only traces of free quinol are excreted

3 Resorcinol is excreted as monoglucuronide (52 %), monosulphate (13.5 %) and in the free state (11.4 %)

4 Quinol and resorcinol monoglucuronides were isolated and characterized as the crystalline methyl esters of the acetoxyphenyltriacyl glucuronides

5 Neither quinol nor resorcinol is oxidized *in vivo* to trihydric phenols or other substances

6 The results have been discussed in relation to the metabolism of benzene, phenol and catechol

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Studies in Detoxication

22 THE METABOLISM OF PHENACETIN (*p*-ETHOXYACETANILIDE) IN THE RABBIT AND A FURTHER OBSERVATION ON ACETANILIDE METABOLISM

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We have shown that acetanilide is converted in the rabbit mainly into the glucuronide and ethereal sulphate of *p*-acetamidophenol (Smith & Williams, 1948), not more than 6–7% being excreted as compounds containing free aromatic amino groups. Phenacetin has antipyretic and analgesic properties similar to those of acetanilide, but is less toxic. The similar pharmacological properties suggest that phenacetin may give rise to the same metabolites as acetanilide, and in this paper we shall show that this suggestion is true.

Earlier workers showed that phenacetin caused an increased output of ethereal sulphate in man, the dog and the rabbit (Muller, 1888, Mörner, 1889, Baccarani, 1900, Hinsberg & Kast, 1887, Mahnert, 1888) and Mörner isolated potassium *p*-acetamidophenylsulphate from the urine of patients receiving phenacetin orally. The presence of a glucuronide and of compounds of *p*-aminophenol in phenacetin urine from man, the dog and the rabbit was also suggested by this early work. According to Muller (1888) no unchanged phenacetin is excreted, but Mörner (1889) found small amounts of phenetidine in human urine. Hinsberg & Kast (1887) observed that a dog which had received 3 g of phenacetin orally excreted urine which strongly reduced alkaline copper solutions, the significance of this observation will be discussed later.

EXPERIMENTAL

The quantitative analyses carried out on phenacetin urine are quoted in the succeeding paper (Smith & Williams, 1949a), in which analytical figures for a number of related aromatic amines are discussed together. At this point it may be mentioned that phenacetin undergoes only a very slight deacetylation in the rabbit and it is to be expected, therefore, that the major metabolites will be aromatic acetamido compounds.

The nature of phenacetin urine The urine of rabbits receiving 0.3–0.7 g/kg phenacetin orally had pH c 8 and was normal in appearance. It did not reduce Benedict's reagent immediately, but a slight reduction was apparent on allowing the test mixture to stand. It gave no colour with FeCl₃ and only a very slight red colour in the diazo test (diazotization and coupling with 1-naphthyl-dimethylamine). The urine yielded no ether soluble material on shaking with ether in a separating funnel.

1 The ethereal sulphate fraction of phenacetin urine

(a) Detection of free *p*-phenetidine

A 24 hr urine (1 l), collected after the feeding of 15 g phenacetin, was evaporated *in vacuo* at 40–50° to 200 ml. Phenetidine was detected in the distillate by the permanganate like colour it gives with FeCl₃, but it could not be isolated in sufficient amount for identification. The concentrated urine was saturated with (NH₄)₂SO₄ and then extracted with 2 × 200 ml portions of acetone. The acetone extract was separated (leaving an aqueous layer *G* containing glucuronide, see section 2 (b) (i)), neutralized with solid K₂CO₃, filtered and reduced to 50 ml *in vacuo*. The residue *B* was shaken with 500 ml dry acetone and the supernatant layer separated and reduced to 50 ml (*C*) *in vacuo*. *C* was now extracted with ether and the extract on evaporation yielded a small oily residue (*D*) which appeared by colour tests to be phenetidine. *D* was dissolved in 1 ml 2*N* HCl, filtered, neutralized with dilute Na₂CO₃ solution and extracted with ether. The extract was evaporated, and the residue treated with a little acetic anhydride and dilute Na₂CO₃ solution. Phenacetin separated and, recrystallized from a little hot water, had *m p* and mixed *m p* 132° (yield, 10 mg, or 0.07% of dose).

(b) Isolation of *p*-aminophenol

The ether extracted concentrate *C* (above) was a syrupy liquid. It was practically free of glucuronides and gave no precipitate with BaCl₂ until boiled with dilute HCl. It did not reduce ammoniacal AgNO₃ and gave no colour in the diazo test, but both these tests became positive after hydrolysis. This fraction, therefore, contained the ethereal sulphate of an acetylated aminophenol.

A 30 ml portion of *C* was boiled for 10 min with 3 ml of conc HCl. The mixture was cooled and extracted with ether. The extract was evaporated to 5 ml and stirred with dilute Na₂CO₃ solution and a little acetic anhydride. The solid which separated was recrystallized from benzene and the crystals (plates) obtained were identified as *ON*-diacetyl *p*-aminophenol, *m p* and mixed *m p* 150° (Found N, 7.3. Calc for C₁₀H₁₁O₄N N, 7.25%). The yield was 120 mg, or 1.3% of dose. The ethereal sulphate fraction, therefore, contained *p*-acetamidophenylsulphuric acid.

2 The glucuronide fraction of phenacetin urine

(a) Isolation of *p*-acetamidophenylglucuronide

The 24 hr urine (400 ml) from two rabbits, which had each received 2 g (0.7 g/kg) of phenacetin, was acidified

with a little glacial acetic acid and, treated with 100 ml saturated lead acetate solution. The precipitate was discarded. The filtrate was made just alkaline with ammonia (sp gr 0.88) and the precipitate which formed was collected, washed well with water, then suspended in water and treated with a current of H_2S to remove Pb. After filtering from PbS, the filtrate was reduced *in vacuo* at 45° to 5 ml. Addition of ethanol threw down a rosy precipitate which dissolved on warming and came down again on cooling as a gum containing some crystals. The whole was, therefore, reduced *in vacuo* to 5 ml and then mixed with 4 ml of benzylamine. This mixture was now diluted to 1 l by alternate additions of ethyl acetate and ethanol so that the last addition of ethyl acetate produced a slight cloudiness. After keeping at 0° for 3 hr the crystalline precipitate (1.65 g) was filtered off and the mother liquor diluted to 2 l with ethyl acetate. On keeping overnight at 0° a further 0.65 g of crystals was obtained. Concentration and redilution of the mother liquor yielded a further 0.6 g. These crystals were identified as the benzylamine salt of *p*-acetamidophenylglucuronide, mp and mixed mp $195-197^\circ$ after recrystallization from aqueous ethanol, $[\alpha]_D^{15} -60^\circ$ (c, 1.4 in water) (Found N, 6.2. Calc for $C_{21}H_{23}O_8N_2 \cdot H_2O$ N, 6.2%). (See Smith & Williams, 1948.) The yield corresponded to 32% of the dose of phenacetin, quantitative estimations showed that 47% of phenacetin is excreted as a glucuronide (Smith & Williams, 1949a).

(b) *The detection of a labile glucuronide*

In the succeeding papers (Smith & Williams, 1949a, b) it is suggested that the main metabolites of aniline and phenetidine in the rabbit are labile glucuronides. Now both acetanilide (Smith & Williams, 1948) and phenacetin undergo a very slight deacetylation in the rabbit and should, therefore, give rise to small amounts of these labile glucuronides. These substances can be detected in urine because they yield, in the presence of *p*-toluidine and NH_4^+ ions, a crystalline complex of *p*-toluidine and ammonium glucuronate. The nature of this complex is discussed by Smith & Williams (1949b).

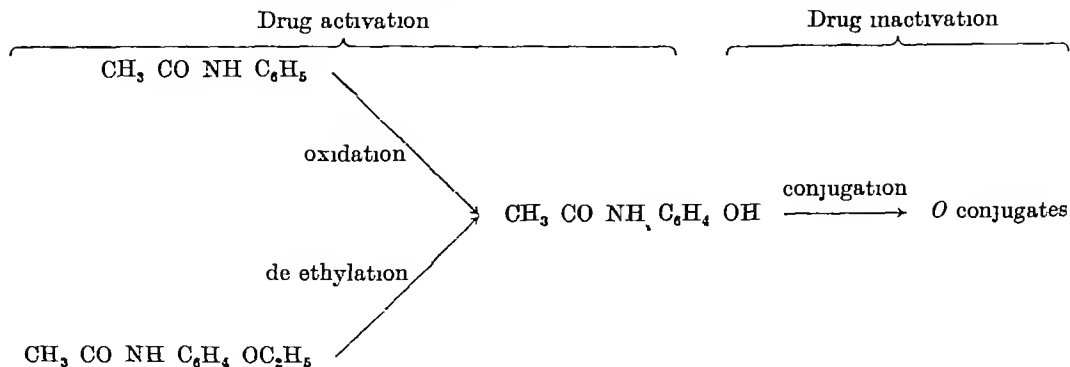
(i) *Detection of a labile glucuronide in phenacetin urine*
The aqueous residue G (see Section 1 (a)) left after removal of the sulphate fraction from phenacetin urine by acetone consisted of 50 ml of urine saturated with $(NH_4)_2SO_4$. Into this was stirred 1 g of *p*-toluidine dissolved in a little

were recrystallized from water and had mp $125-128^\circ$, not depressed on admixture with the *p*-toluidine ammonium glucuronate complex from aniline or phenetidine urine. The compound rapidly reduced Benedict's reagent and gave the Tollens naphthoresorcinol reaction very readily.

(ii) *From acetanilide urine* A 24 hr urine (800 ml) from rabbits which had collectively received 10 g acetanilide was reduced *in vacuo* at $40-50^\circ$ to 160 ml. The concentrate was acidified with a little 2N-HCl, saturated with $(NH_4)_2SO_4$ and extracted with acetone (2×150 ml). The aqueous layer was separated and treated with an ethanolic solution of 5 g *p*-toluidine followed by 50 ml ethanol. On keeping this mixture, solid $(NH_4)_2SO_4$ separated and settled on the bottom of the vessel whereas crystals of the *p*-toluidine ammonium glucuronate complex collected at the ethanol-water interface. The complex was collected and recrystallized from water (yield, 30 mg or 0.1% of dose). It had mp and mixed mp $125-128^\circ$ with the corresponding product from aniline urine (Found N, 9.6. $C_{20}H_{21}O_7N_2$ requires N, 9.9%). It gave a characteristically rapid Tollens reaction and reduced Benedict's solution readily on warming.

DISCUSSION

The present work shows that the main metabolites of phenacetin in the rabbit are the same as those of acetanilide, i.e. *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid, and quantitatively these account for 54% (47% as glucuronide and 7% as ethereal sulphate) of the phenacetin fed. These metabolites are produced in roughly the same ratio as found in *p*-acetamidophenol or acetanilide metabolism, for the glucuronide/ethereal sulphate ratio for phenacetin is 6.9, for *p*-acetamidophenol, 6.3 and for acetanilide, 5.8 (see Smith & Williams, 1948). From our present results and those on acetanilide it appears that phenacetin and acetanilide owe their therapeutic activity to their metabolic conversion to *p*-acetamidophenol (cf Hinsberg & Treupel, 1894). This phenol is probably then inactivated by conjugation with glucuronic and sulphuric acids. In the case of acetanilide the process of activation involves oxidation, whereas with phenacetin it involves de-ethylation thus

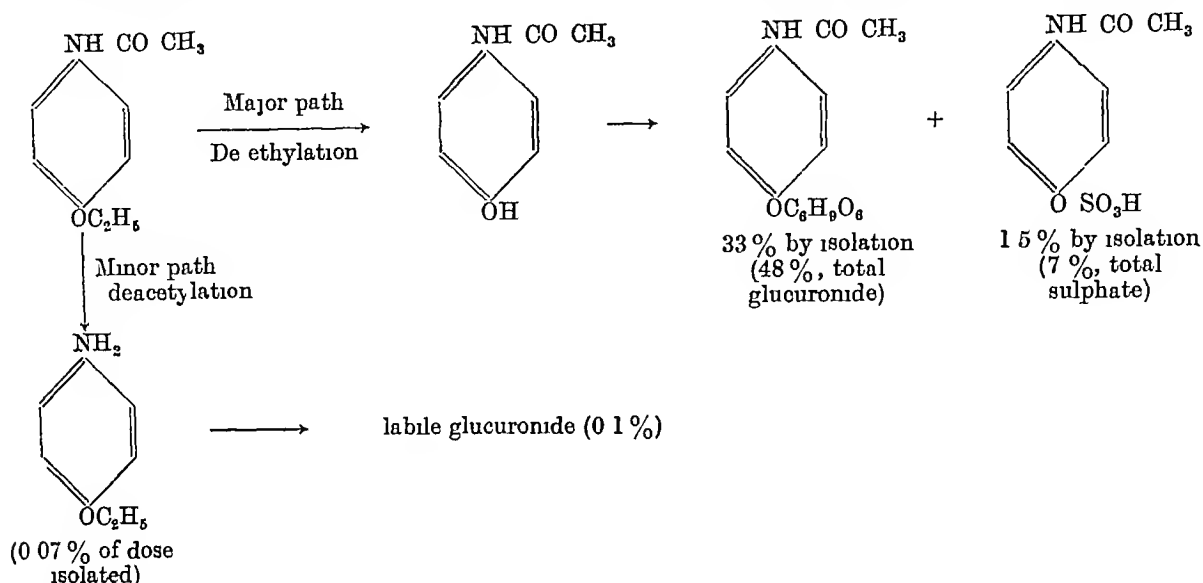


acetone. After several hours the crystalline precipitate which had separated was filtered off and washed with acetone to remove excess *p*-toluidine. The crystals (50 mg)

Phenacetin is more satisfactory as an analgesic and antipyretic drug than acetanilide. It has been suggested that the rate at which a drug of this group

is metabolized (presumably to *p* acetamidophenol) depends on its molecular size (Hinsberg & Treupel, 1894). Thus molecules smaller than phenacetin are rapidly metabolized and are consequently more toxic, whereas with larger molecules such as the propyl and amyl analogues of phenacetin, the change is probably slower and, while toxic effects are less marked, there is also a loss of therapeutic effect (see Gaddum, 1944).

we have not yet elucidated. These glucuronides are relatively easy to detect for they readily decompose yielding free glucuronic acid which forms a characteristic crystalline complex in the presence of *p* toluidine and ammonium ions. This complex was isolated from both phenacetin and acetanilide urines, the yields corresponding to 0.1% of the dose in each case. It was suggested in an earlier paper (Smith & Williams, 1948) that the metabolism of



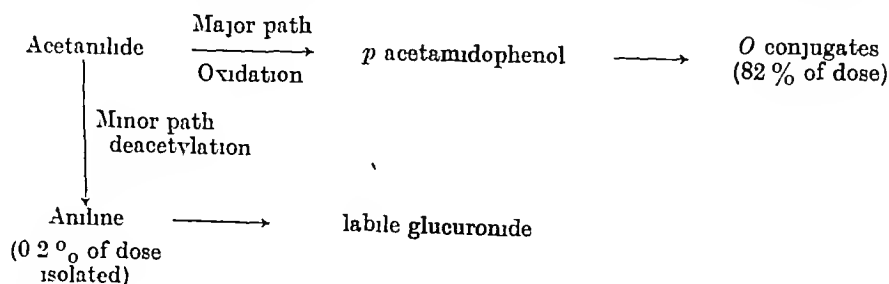
If it is conceded that both the therapeutic and toxic effects of acetanilide and phenacetin are due to the metabolic formation of *p* acetamidophenol, then the greater efficiency and lesser toxicity of phenacetin are due to its de ethylation being a slower process than the oxidation of acetanilide. The toxic effects of phenacetin are also less than those of acetanilide because the slower rate of production of *p* acetamidophenol permits a more efficient detoxication by conjugation.

The extent of deacetylation of phenacetin in the rabbit is very small, but we were able to detect traces of phenetidine in the urine. This small amount of phenetidine should give rise to phenetidine metabolites. Similarly, acetanilide is slightly deacetylated by the rabbit, and should, therefore, give rise to small amounts of aniline metabolites. Now the major metabolites of both aniline and phenetidine in the rabbit are labile glucuronides whose structure

acetanilide would be similar to that of aniline in animals capable of extensively deacetylating the aromatic acetamido group. Such animals are the dog, cat, and pigeon (Krebs, Sykes & Bartley, 1947). As mentioned earlier Hinsberg & Kast (1887) observed that a dog receiving phenacetin excreted a strongly reducing urine. This observation can now be explained, for the dog probably converts phenacetin to a considerable extent to phenetidine which may then give rise to the labile glucuronide which we have found to be the major metabolite of phenetidine in the rabbit.

The main features of the metabolisms of phenacetin in the rabbit are as shown above. These findings are probably applicable to man but not to the pigeon, dog and cat.

In the case of acetanilide, a minor pathway of metabolism can now be added to the results given earlier (Smith & Williams, 1948).



SUMMARY

1 The fate of phenacetin has been studied in the rabbit and it has been found to be largely transformed into *p* acetamidophenylglucuronide and *p* acetamidophenylsulphuric acid, these occurring in the urine in the ratio 6.9 : 1. The major metabolic change undergone by phenacetin is, therefore, deethylation, followed by conjugation.

2 Deacetylation of phenacetin takes place only to a very minor extent. This was shown by the detection of traces of free *p*-phenetidine in the urine, and the isolation of minute amounts of a crystalline

complex of *p* toluidine and ammonium glucuronate derived from a labile glucuronide which is a major metabolite of *p*-phenetidine in the rabbit.

3 Acetanilide is also slightly deacetylated, for acetanilide urine also contains traces of a labile glucuronide which is a major metabolite of aniline (see Smith & Williams, 1949*a*).

4 The results obtained have been correlated with the known therapeutic and toxic effects of phenacetin and acetanilide.

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Studies in Detoxication

23 THE FATE OF ANILINE IN THE RABBIT

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In the past numerous investigations (see summary by Gross, 1946) have been carried out on the fate of acetanilide in the body, but few on aniline. This is probably the result of the belief that aniline and acetanilide are interconvertible *in vivo*. We have now found that in the rabbit this is not wholly true. In this animal acetanilide is almost entirely converted into the glucuronide and ethereal sulphate of *p* acetamidophenol, the excretion of compounds containing free diazotizable amino groups being about 6–7 % of the dose (Smith & Williams, 1948*a*). Furthermore, *p* substituted acetanilides are either excreted completely unchanged or deacetylated only to a very small extent (Smith & Williams, 1948*b*, 1949*a*). In this paper we shall show that aniline gives rise to metabolites different from those of acetanilide.

The recent work of Krebs, Sykes & Bartley (1947) has shown that the extent of deacetylation of the aromatic acetamido group depends on animal species, deacetylation being extensive in the cat, dog and pigeon, but very small in man and the rabbit. From this it follows that aniline and acetanilide are likely to give rise to similar metabolites in animals such as the dog and cat, but to different ones in man and the rabbit.

Earlier work on the fate of aniline appears to be very scanty. Muller (1887) studied a human case of poisoning by 25 g of aniline, and found that the urine reduced Fehling's solution and contained conjugated *p*-aminophenol. Schmiedeberg (1878) fed dogs with aniline acetate and identified *p* aminophenol in the urine after acid hydrolysis. According to Elson, Goulden & Warren (1946) rats probably excrete aniline as the ethereal sulphate of *p* aminophenol. It is clear from this earlier work that conjugated *p* aminophenol is a metabolite of aniline, but we shall show that in the rabbit this phenol is not a major metabolite.

The study of the metabolic fate of aniline is not only of considerable theoretical interest, but it is also important because of the possible role of aniline as a bladder carcinogen (for discussion see Goldblatt, 1947).

I QUANTITATIVE INVESTIGATION OF TYPES OF COMPOUNDS EXCRETED

EXPERIMENTAL

Glucuronic acid and ethereal sulphate in urine were determined as described in earlier papers from this laboratory (e.g. Smith & Williams, 1948*a*).

Diazo test Throughout this paper this term means diazotization with 1% NaNO₂ and dilute HCl, then addition of 1% ammonium sulphamate followed by coupling with 1% ethanolic 1 naphthylmethanamine

Free aromatic amino groups were determined using a modification of the Bratton & Marshall (1939) procedure for sulphanilamide. Three compounds, namely, aniline hydrochloride and *o* and *p* aminophenylglucuronides (Williams, 1943) were used as standards. If a dilute solution of any of these three compounds is diazotized and coupled with *N*-(1 naphthyl)ethylenediamine as in the Bratton & Marshall procedure a red (aniline and *o* aminophenylglucuronide) or red purple (*p* aminophenylglucuronide) colour develops gradually and reaches a stable intensity in 24 hr. A similar slow rate of colour development was also found when 1 naphthylmethanamine and sulphatoethyltoluidine were used as coupling agents, but they were not as sensitive as naphthylethylenediamine. In order to obtain reproducible results diazotization had to be prolonged for 10 min at 20° before adding the coupling agent, and furthermore it was found that the final colour intensity depended on the concentration of acid in the solution, a point which had to be considered in the construction of calibration curves for 'free' and 'total' amino groups.

The procedure finally adopted was as follows. The urine was diluted so as to contain an equivalent of 1–8 mg/l of aniline. 10 ml of diluted urine were mixed with 0.5 ml 4*N* HCl followed by 1 ml 0.1% NaNO₂ and allowed to stand 10 min at room temperature, then 1 ml of 0.5% ammonium sulphamate was added followed 2 min later by 1 ml of 0.1% aqueous *N*-(1 naphthyl)ethylenediamine hydrochloride. After mixing, the solution was left for 24 hr, then made up to 25 ml with water, and the colour measured with a Spekker photoelectric absorptiometer using an Ilford no 605 yellow green filter. The readings were then referred to calibration curves constructed in the same way, but using standard aqueous solutions of aniline hydrochloride, *o* or *p* aminophenylglucuronide instead of urine. The *o* glucuronide gives the same red colour as does aniline, the colour ratio *o* glucuronide/aniline being 1.04. The *p*-glucuronide gives a slightly different colour (red purple), and using the same filter (no 605) the colour ratio *p* glucuronide/aniline = 0.84. In this procedure the free *o* and

p aminophenols do not yield interfering colours, and if present in urine are not estimated.

'Total' aromatic amino groups were estimated by the same general procedure. In this case 10 ml of diluted urine were mixed with 1.5 ml 4*N* HCl and heated for 1 hr in a boiling water bath. After cooling the solution, the procedure was as indicated above, except that the absorptiometer readings were referred to calibration curves constructed using 1.5 ml 4*N* HCl instead of 0.5 ml. By this procedure acetanilide in pure solution could be determined almost quantitatively. Under the hydrolysis conditions used, *o* aminophenylglucuronide is only hydrolyzed to a slight extent, for the colour ratio *o* aminophenylglucuronide/aniline changes only from 1.04 to 0.91. With *p* aminophenylglucuronide hydrolysis of the glucuronidic link proceeds further for the colour ratio *p* aminophenylglucuronide/aniline changes from 0.84 to 0.59.

Owing to the variable extents of hydrolysis of the conjugated metabolites encountered in the urines examined, the estimations of 'total' aromatic amino groups are not easily interpreted, and, therefore, are quoted (in Table 1) with reserve. The estimations of 'free' aromatic amino groups, however, are more capable of interpretation.

RESULTS

The excretion of conjugated glucuronic acid and ethereal sulphates and of diazotizable amino groups by rabbits receiving oral doses of aniline, phenetidine, *o*- and *p*-aminophenols and their *N*-acetyl derivatives are given in Table 1.

The results for the acetylated compounds are more easily interpreted than those for the free amino compounds because the qualitative results (Smith & Williams, 1948*a*, 1949*a*) show that the metabolism of the acetyl derivatives is the less complicated, they do not undergo deacetylation to any great extent.

For the acetyl compounds the percentage of total diazotizable amino groups (Table 1, column 7) is expected to be only slightly less than the percentage of glucuronide (column 3), for the following reason.

Table 1 The excretion of conjugated glucuronic acid and sulphate and of diazotizable amino groups by rabbits receiving aniline and its derivatives orally (averaged results)

Compound fed	Dose (mg/kg)	Percentage excreted				
		As glucuronide (G)	As etheral sulphate (E)	G/E	Containing diazotizable amino groups	
					Free	After acid hydrolysis
Aniline	200	70	28	2.5	40*	60*
<i>p</i> Ethoxyaniline (phenetidine)	300	120	30	4.0	3†	25†
<i>p</i> Aminophenol	250	43	18	2.5	28†	45†
<i>o</i> Aminophenol	210	(28)‡	25	1.1	33§	42§
Acetanilide	250	70	12	5.8	6,* 7†	37,* 61†
<i>p</i> Ethoxyacetanilide (phenacetin)	350	48	7	6.9	0†	49†
<i>p</i> Acetamidophenol	280	63	10	6.3	0†	49†
<i>o</i> Acetamidophenol	280	39	20	2.0	3.5§	33§

* Aniline as standard

† This figure will be too low because *o* aminophenylglucuronide cannot be estimated by the naphthoresorcinol method

‡ *o* Aminophenylglucuronide as standard

§ *p* Aminophenylglucuronide as standard

Qualitative results show that the main glucuronide excreted after administration of acetanilide, phenacetin and *p*-acetamidophenol is *p*-acetamidophenylglucuronide and the ethereal sulphate is *p*-acetamidophenylsulphuric acid. Under the conditions of hydrolysis for the estimation of total amino groups the ethereal sulphate is completely hydrolyzed to *p*-aminophenol which gives no colour in the estimation procedure, the glucuronide, however, is deacetylated to *p*-aminophenylglucuronide which is only slightly hydrolyzed to *p*-aminophenol. Thus the amount of *p*-aminophenylglucuronide produced by hydrolysis, and estimated by diazotization and coupling should be only slightly less than the amount of *p*-acetamidophenylglucuronide estimated by the naphthoresoreinol reaction in the unhydrolyzed urine.

An examination of the figures for *p*-aminophenol suggests that both *p*-acetamido- and *p*-aminophenylglucuronide are its metabolites, because the figure for the total diazotizable amino groups (45%) is almost identical with the figure for glucuronide output (43%), and there is an increase in diazotizable amino groups on acid hydrolysis from 28 to 45%. This increase must be due to hydrolysis of the *p*-acetamido- to the *p*-amino glucuronide, because hydrolysis of the ethereal sulphate will give *p*-aminophenol which has no effect on the diazo reaction.

In the case of *o*-aminophenol, the total glucuronide formed is probably more accurately given by the figure for the total amino groups (42%), the figure of 28% obtained by the naphthoresoreinol method is definitely too low because Hanson, Mills & Williams (1944) have shown that *o*-aminophenylglucuronide is not completely hydrolyzed under the conditions of their method for estimating glucuronic acid.

II THE ISOLATION OF ANILINE METABOLITES

EXPERIMENTAL

1 The effects of aniline on rabbits

Each dose (c. 0.5 g/kg in most cases) was dissolved in 20 ml water containing just enough HCl to give a clear solution. The amount of HCl used was less than that required to form the hydrochloride and this solution was better tolerated than aniline hydrochloride. Single doses of 1.3 g/kg were not fatal in this form, but doses of 0.3 g/kg repeated daily for 4 days were fatal. Aniline had no narcotic effect similar to that of acetanilide (Smith & Williams, 1948a).

2 The nature of aniline urine

The urine had a dark colour but a normal pH of 8. This colour did not appear to be due to porphyrins, for the urine under ultraviolet light showed not a red but a pale green fluorescence. The urine readily reduced Benedict's solution and the reducing substance was not glucose (osazone test).

No reducing substance could be extracted by ether, butanol or amyl alcohol at different values of pH.

The urine from two rabbits, each of which had received 3 g of aniline, was found to contain 4.4 g extra glucuronic acid (estimated by the method of Hanson *et al* 1944). By titration with Benedict's quantitative reagent the urine was found to contain 3.8 g of reducing material, calculated as glucuronic acid. From this it can be deduced that aniline urine contains at least two glucuronides, one reducing and present in large amount, and the other non-reducing. The urine gave a strong red colour in the diazo test thus showing the presence of free aromatic amino groups. The Tollens test for glucuronic acid was given very readily, for a deep blue precipitate was formed even before the mixture had reached boiling point. These tests suggested that aniline urine may contain a very labile, reducing glucuronide. Neither the urine itself nor ether extracts of it gave any colour with FeCl₃ and from this it could be concluded that free aminophenols were not present. The urine was slightly laevorotatory, filtered and clarified with a few drops of HCl it gave $\alpha_D - 0.18^\circ$ in a 1 dm tube. From a knowledge of the extra glucuronic acid content (0.8 g/100 ml) of this urine and assuming that the glucuronide present is *p*-aminophenylglucuronide α_D for 1 dm should be -0.64° (see Williams, 1943). Thus the conclusion can be drawn that the main glucuronide is not that of *p*-amino phenol.

3 Extraction of aniline urine with ether Isolation of unchanged aniline

(a) *At acid reaction* Continuous ether extraction of acidified aniline urine yielded no material derived from aniline.

(b) *At alkaline reaction* A 24 hr urine (1200 ml), collected after feeding 12 g aniline, was treated with a few ml of 2N KOH and extracted continuously with ether for 1 hr. The extract, dried over Na₂SO₄, was evaporated, leaving 0.42 g of a dark oil which was identified as aniline by preparing from it tribromoaniline (m.p. and mixed m.p. 117–118°) and benzanilide (0.76 g, m.p. and mixed m.p. 160°). In other experiments the dried ether extract was treated with a saturated ethereal solution of oxalic acid and the crystalline precipitate of aniline hydrogen oxalate (m.p. 158°, Anselmino (1903) gives m.p. 163°) collected, dried and weighed. The recoveries of free aniline in five experiments were 3, 5, 6, 9 and 9.5% (average 6.5%) of the dose. Neither acetanilide nor aminophenol was found.

(c) *The origin of the free aniline* The aniline isolated could either be free, unchanged aniline or it could arise by decomposition of a labile precursor. That the second possibility was unlikely was shown as follows. 12 g of aniline were fed to six rabbits and the urine (950 ml) collected for 24 hr. To 350 ml of the urine were added 10 g of Na₂CO₃ and the whole was extracted with ether for 4 hr. On addition of ethereal oxalic acid to this extract there was obtained 0.8 g of aniline hydrogen oxalate. Another 350 ml of the urine was treated in a similar manner except that, before ether extraction, the alkaline urine was boiled vigorously for 10 min. The yield of aniline hydrogen oxalate was again 0.8 g. In another experiment 400 ml of aniline urine were made alkaline and exhaustively extracted with ether to remove free aniline. To the urine were now added 40 ml of conc. HCl and the whole was boiled for 20 min. The hydrolyzed urine was cooled, neutralized with 40% NaOH,

made alkaline with solid Na_2CO_3 and extracted exhaustively with ether. Although this extract contained aminophenols (see below) it contained no trace of aniline.

These experiments show that the aniline present in the urine probably occurs as such for there is no increase in the amount present when the urine is heated with acid or alkali.

4 The ethereal sulphate fraction of aniline urine *Isolation of o- and p aminophenols and detection of 4 aminoresorcinol*

(a) *Mild hydrolysis of aniline urine* Aniline urine (400 ml from 5.8 g aniline) was made alkaline with a little 2N NaOH and the free aniline removed with ether as described above. It was then acidified with 40 ml conc HCl and boiled for 20 min. After cooling, it was neutralized with NaOH, made alkaline with 1 g solid Na_2CO_3 and continuously extracted with ether for 1.5 hr. The extract was reduced to 10 ml and the crystals (60 mg) which separated collected. They had m.p. 180° , gave a purple colour with FeCl_3 and were identified as *p* aminophenol by conversion to *ON* dibenzoyl *p* aminophenol, m.p. and mixed m.p. 230° (yield, 0.9% of the dose). The filtrate was reduced to 0.5 ml on the water bath and on cooling 50 mg of yellow plates separated. These were collected and found to give a red colour with FeCl_3 and an intense yellow colour with nitrous acid, but no immediate red colour in the diazo test. They were identified as *o* aminophenol by conversion into *ON*-dibenzoyl *o* aminophenol, m.p. and mixed m.p. 178 – 179° (yield, 0.7% of the dose). The residue, which remained after separation of the *o* and *p* aminophenols, gave colour reactions for 4 aminoresorcinol, for on shaking in air in the presence of NaOH it gave an intense blue colour which on standing faded to green and finally (1 hr) to brown (Henrich & Wagner, 1902).

We prepared 4 aminoresorcinol hydrochloride by the method of Henrich & Wagner (1902) and carried out trial separations of the three phenols, but we failed to isolate the labile 4 aminoresorcinol from aniline urine in pure crystalline form. When fed to a rabbit, 4-aminoresorcinol (0.5 g) causes the excretion of a urine which is almost black in colour.

(b) *Acetone ammonium sulphate fractionation of aniline urine* A total of 8 g aniline was fed to four rabbits. The 24 hr urine (800 ml) was evaporated *in vacuo* at 40 – 45° to 100 ml. The concentrate was acidified with a little HCl, saturated with $(\text{NH}_4)_2\text{SO}_4$ and then shaken with 250 ml acetone. The ethereal sulphates present, together with some glucuronides, passed into the acetone layer. The main bulk of the labile glucuronide (see p. 247) and other material reacting with naphthoresorcinol remained in the aqueous layer. The acetone extract was made alkaline with solid K_2CO_3 and concentrated to a small volume *in vacuo* at 40 – 50° . Dry acetone was now added and a small flocculent glucuronide containing precipitate which rapidly turned into gum was deposited on standing. After removal of this gum, the acetone solution gave only a weak naphthoresorcinol reaction and contained no inorganic sulphate, but gave strong tests for ethereal sulphate. It was taken to small bulk *in vacuo*. Attempts to crystallize the organic sulphate or prepare crystalline derivatives such as benzyl amine salts were unsuccessful. The concentrate gave strong tests for free amino groups and ethereal sulphates, but did not reduce ammoniacal AgNO_3 . It was, therefore, acidified

with 0.1 vol conc HCl and boiled for 10 min to hydrolyze the ethereal sulphates and then diluted to 50 ml with water. The solution now reduced ammoniacal AgNO_3 and gave an intense blue colour on shaking in air with NaOH solution, indicating the presence of 4-aminoresorcinol. The main bulk of the hydrolyzed solution was extracted with 2×100 ml ether. Evaporation of the ether left a small amount of dark tar which gave no diazo or FeCl_3 test. The residual solution was, therefore, made alkaline with solid Na_2CO_3 and extracted with 2×100 ml ether. From this extract *p* aminophenol (3.1% of the dose) and *o* aminophenol (0.3% of the dose) were isolated and identified as before.

The residue after separation of these two phenols left on evaporation a small tarry residue giving a faint red colour in the diazo test. On shaking the tar in air with NaOH solution an intense blue colour was obtained which faded to brown in about an hour. 4-Aminoresorcinol was thus present, but attempts to obtain crystalline derivatives were unsuccessful.

5 The glucuronide fraction of aniline urine

(a) *Preparation of the glucuronide gum* 12 g of aniline were fed to eight rabbits and the urine was collected for 24 hr. Free aniline (3% of the dose) was removed with ether as described in section 3(b). The alkaline urine was now made faintly acid and lead acetate added until no further precipitation occurred. The precipitate was removed and the filtrate was neutralized with ammonia and an excess of saturated basic lead acetate added. The precipitate was filtered, washed with water, and then suspended in water and Pb removed with H_2S . The Pb free filtrate was dried *in vacuo* at 40 – 50° to a reddish gum (24 g) which was purified by dissolving in the minimum of water and pouring into ethanol. A precipitate of inorganic material separated and after filtration the solution was concentrated *in vacuo* at 40 – 50° to a gum which reduced Benedict's solution on warming, gave a weak red colour in the diazo test, and reacted very rapidly in the Tollens test for glucuronic acid. The gum could not be induced to crystallize and its potassium, brucine, *o* toluidine and benzylamine salts separated from ethanol as flocculent precipitates which readily formed gums. It contained no other soluble material.

(b) *Isolation of p aminophenol from the gum* The purified glucuronide gum obtained after feeding 5 g of aniline was dissolved in 150 ml 5N H_2SO_4 , filtered and then boiled for 2.5 hr. The dark hydrolysate, after cooling and making alkaline, was extracted for 3 hr with ether which on evaporation left a crystalline residue from which 150 mg (5% of the dose) of *p* aminophenol, m.p. 182 – 184° , were obtained. No other phenol was identified in the hydrolysate.

(c) *Methylation and acetylation of the gum* *Isolation of p acetamidophenyltriacetylglucuronide methyl ester* Methylation of the gum with dimethyl sulphate and alkali followed by Ag_2O and methyl iodide yielded no crystalline product. A similar result was obtained on acetylation of the gum with pyridine and acetic anhydride. A crystalline derivative was, however, obtained by the following procedure. 2 g of the gum dissolved in 10 ml absolute ethanol were treated with an excess of diazomethane in 100 ml dry ether, a small precipitate appeared. After standing for 24 hr at room temperature the precipitate had partly dissolved again.

and the whole was evaporated *in vacuo*. The product, a brown gum which would not crystallize, was dissolved in 30 ml of a pyridine acetic anhydride mixture (1:1) and kept overnight. It was then poured into 200 ml water and the solution was extracted with chloroform. The extract, washed free of acetic acid with water, gave on evaporation a gum which crystallized from acetone-water mixtures as long needles, m.p. 100° (resolidifying and then melting again indefinitely at 170–180°). This compound appears to be a dimorphic *p*-acetamidophenyltriacylglucuronide methyl ester (yield 70 mg, 1% of the dose). On recrystallization from absolute ethanol it yielded needles, m.p. 205°, not depressed by an authentic sample (Smith & Williams, 1948a). It showed $[\alpha]_D^{20} - 22.4^\circ$ (c, 4 in chloroform) (Found C, 54.3, H, 5.5. Calc for $C_{21}H_{25}O_{11}N$ C, 54.0, H, 5.4%). In another experiment 0.6% of the dose was isolated as this ester.

The two methyl esters, m.p.'s 100 and 205°, have been described by us in an earlier paper (Smith & Williams, 1948a) and we suggested that the former was a hydrate of the latter, but we only quoted analyses for the latter compound. We now find that both give analyses correct for the anhydrous compound and show identical specific optical rotations in chloroform. The ester of m.p. 100° gave the following analysis: Found C, 53.6, H, 5.65, N, 3.2. Calc for $C_{21}H_{25}O_{11}N$ C, 54.0, H, 5.4, N, 3.0%. $[\alpha]_D - 22.1^\circ$ (c, 7 in chloroform). We conclude, therefore, that we have here a case of dimorphism. Neither of the two forms obtained from the aniline gum depressed the m.p.'s of the corresponding authentic forms prepared synthetically from *p*-aminophenylglucuronide.

Our results so far indicate that the gum contains either *p*-aminophenylglucuronide or *p*-acetamidophenylglucuronide, or both, but the small yields of the above derivatives suggest that these glucuronides are not major metabolites.

(d) *Isolation of p-aminophenylglucuronide*. The evidence presented on the nature of the glucuronide gum suggests that it contains at least two glucuronides, viz. large amounts of a labile, reducing glucuronide and the non-reducing *p*-aminophenylglucuronide. Since the ethereal sulphate fraction contains *o*-aminophenol, it is also possible that the gum may contain *o*-aminophenylglucuronide. Attempts were, therefore, made to fractionate the gum.

At first advantage was taken of the fact that the aminophenylglucuronides have isoelectric points where they show minimum solubility. It was found that the *o* compound had a minimum solubility at c.p.H. 3.0–3.5 and the *p* compound at c.p.H. 4.0–4.5. Thus the *p* compound should crystallize best at about p.H. 4.0–4.5.

Aniline urine (140 ml) clarified with 50 ml colloidal iron was evaporated *in vacuo* to 70 ml then buffered to p.H. 4.3 and seeded with *p*-aminophenylglucuronide. No precipitate appeared after 5 days at 0°.

The basic lead acetate precipitate from aniline urine after feeding 10 g of aniline was prepared in the usual manner and Pb removed with H_2S . The filtrate (350 ml) from the PbS had p.H. 2.3. A 50 ml portion of this filtrate was brought to p.H. 4.3 with acetate buffer, but no precipitate was obtained after 2 days at 0°. The rest of the filtrate (300 ml) was made faintly alkaline with ammonia and then treated with cold saturated mercuric acetate solution. A small pinkish precipitate separated and was filtered off. The labile glucuronide appeared in the filtrate together with

the main bulk of the naphthoresorcinol reacting material of the urine. The mercuric acetate precipitate did, however, contain a glucuronide. It was suspended in water and Hg removed with H_2S . The filtrate from the HgS was evaporated *in vacuo* at 40–50° to 15 ml and this was brought to p.H. 4.3 with 0.5M-Na acetate. The mixture was kept at 0° overnight. The crystalline precipitate (180 mg, or 0.6% of the dose) was filtered off, washed with water, ethanol and then ether. It was recrystallized from hot water, from which it formed felted needles, m.p. 215–216°, not depressed by authentic *p*-aminophenylglucuronide (Williams, 1943). It showed $[\alpha]_D^{20} - 83.4^\circ$ (c, 4.2, in 0.5N H_2SO_4) (Found C, 48.2, H, 5.6, N, 4.6. Calc for $C_{12}H_{16}O_7N \cdot H_2O$ C, 47.5, H, 5.6, N, 4.6%). In another experiment, using 12 g aniline, 295 mg (0.8% of the dose) of the glucuronide were isolated.

This experiment was repeated in exactly the same manner, except that the glucuronide gum solution was saturated with SO_2 prior to the Hg precipitation. The yield of *p*-aminophenylglucuronide was not increased, nor was it increased by boiling the urine with hot alkali prior to preparation of the gum. These results indicate that *p*-aminophenylglucuronide occurs in the urine as such and is not derived from a labile glucuronide.

(e) *Isolation of p-acetamidophenylglucuronide*. The glucuronide fraction from the urine (350 ml) of four rabbits which had collectively received 8 g of aniline was prepared as before. The fraction was concentrated to 30 ml of syrupy liquid to which was added, with shaking, 200 ml of absolute ethanol. A reducing gum was precipitated which yielded 7 g (15% of the dose) of the crystalline *p*-toluidine ammonium glucuronate complex (see p. 247). The ethanolic solution after removing the gum was now reduced *in vacuo* to 10 ml, and again precipitated with ethanol to remove further traces of the labile glucuronide. The concentration and precipitation with ethanol was repeated once more. In this way most of the labile glucuronide was removed and the final ethanolic solution was concentrated at 40–50° to a non-reducing gum (2 g) which contained conjugated glucuronic acid, but only gave a diazo test after hydrolysis by acid. The non-reducing gum was now dissolved in 50 ml 95% ethanol and the solution treated with 1 g of benzylamine followed by 400 ml ethyl acetate. After 1 hr at 0° the crystalline precipitate (200 mg, 0.5% of the dose) was collected and recrystallized twice from ethanol and ethanol-ethyl acetate. The crystals were identified as the benzylamine salt of *p*-acetamidophenylglucuronide, m.p. and mixed m.p. 195–198°, $[\alpha]_D^{15} - 59^\circ$ (c, 0.7 in water) (see Smith & Williams, 1948a). A further quantity of this salt was obtained from the mother liquor, but it could not be satisfactorily purified.

We have attempted to assess by various means the amount of *p*-acetamido and *p*-aminophenylglucuronides in aniline urine. By actual isolation we found 0.8% of the dose as *p*-amino and at least 0.5% as *p*-acetamidophenylglucuronide, and by isolation of the acetylated methyl ester, 1% of the dose. These values are obviously minimal. A study of the ultraviolet absorption spectrum of the gum (see p. 247) gave an estimate of 10%. The difference between the 'extra' glucuronic acid content of the acid content of the urine and its reducing power towards Benedict's reagent gave a value of 15%. We can, therefore, conclude that about 10–15% of the aniline fed is excreted as free and acetylated *p*-aminophenylglucuronide.

(f) *The isolation of free D glucuronic acid from the gum* It was mentioned in § 2 that aniline urine had strong reducing properties, Benedict's and Fehling's solution being very readily reduced on warming. Furthermore, the reducing substance was not glucose. The reducing substance appeared in the basic lead acetate precipitate of the urine, and constituted the major fraction of the glucuronide gum prepared from this precipitate. On examination of the gum it was found possible to isolate, without the use of hydrolytic methods, considerable amounts of free glucuronic acid. The significance of this free glucuronic acid is not yet clear to us.

The isolation of glucuronic acid was achieved through the use of *p* toluidine. Glucuronic acid forms in the presence of *p* toluidine and NH_4^+ ions a sparingly soluble, crystalline compound whose components are two molecules of *p* toluidine, and one of ammonium glucuronate. It appears to be a convenient compound for isolating free glucuronic acid, but it is not formed from the lactone, glucurone. The preparation of authentic samples of this compound, and of the corresponding compound from *p* galacturonate together with a discussion of their nature are described in the succeeding paper (Smith & Williams, 1949b).

The isolation of the compound from aniline urine was achieved as follows. A solution of 8 g. of the purified glucuronide gum (see § 5 (a)) dissolved in 5 ml. cold water was mixed with a suspension of 4 g. *p* toluidine in 1 ml. ethanol and 4 ml. warm water. The toluidine dissolved on stirring, and, on cooling, the mixture crystallized. After keeping at 0° for a few hours the crystals were filtered at the pump, washed with a little ethanol followed by a large volume of ether (yield, 7 g. this corresponds to 21% of the dose of aniline, assuming that one molecule of aniline gives rise to one of glucuronic acid). The *p* toluidine ammonium glucuronate complex, recrystallized from hot dilute ethanol, formed colourless rectangular plates, m.p. 125–128° (decomp.), $[\alpha]_D^{20} + 20^\circ \rightarrow +11^\circ$ (constant value in *c* 3 hr.) (*c*, 5 in 0.4N HCl). Ultraviolet absorption spectrum in water, λ_{max} 234 m μ , ϵ_{max} 16,900, λ_{max} 285 m μ , ϵ_{max} 2700, in N HCl, λ_{max} 264 m μ , ϵ_{max} 400. Elementary analysis suggested that two hydrates of this complex existed (cf. the hydrates of glucose toluidide, Irvine & Gilmour, 1909). The one less frequently obtained contained 0.5 H₂O more than the other (Found (1) C, 55.5, H, 7.2, N, 9.6. C₂₀H₂₅N₃O₆ · 1.5H₂O requires C, 55.3, H, 7.4, N, 9.7%. (2) C, 56.7, H, 7.2, N, 9.25, glucuronic acid, 47.6 C₆H₈O₇ · H₂O requires C, 56.45, H, 7.3, N, 9.9, glucuronic acid, 45.7%). The compound quickly reduced Benedict's reagent on warming and gave the Tollens test for glucuronic acid very rapidly. With Nessler's reagent it gave a brown precipitate, a reaction not given by *p* toluidine.

(g) *Spectroscopic observations on the glucuronide gum* The isolation of free glucuronic acid from the gum raises the question of its source. It could arise from a labile glucuronide of aniline metabolism or aniline may stimulate the excretion of free glucuronic acid. We thought that spectroscopic examination of the gum might give us information on the first of these alternatives. A possible glucuronide would be the unknown β phenylhydroxylamine glucuronide. This compound would probably be labile and break up to phenylhydroxylamine which would rapidly form azoxybenzene. The latter can be detected by its characteristic ultraviolet absorption. When fed to rabbits, β phenylhydroxylamine (200 mg.) does not cause the excretion of a reducing urine.

The glucuronide gum for spectroscopic examination was obtained by working up the 24 hr. urine of a rabbit which had received 2 g. aniline (see § 5 (a)). It was dissolved in 25 l. 50% aqueous ethanol and the solution examined with a Hilger E 3 spectroscope. The absorption spectra of the gum solution, *p* aminophenylglucuronide, *p* acetamidophenylglucuronide and azoxybenzene are reproduced in Fig. 1.

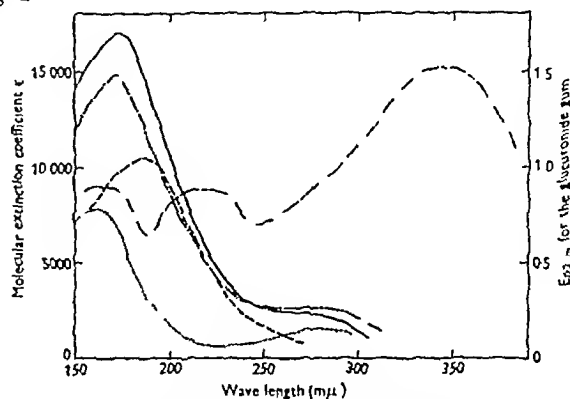


Fig. 1 The ultraviolet absorption spectrum of the glucuronide gum from aniline urine compared with the spectra of possible components — — —, the glucuronide gum (after feeding 2 g. aniline) dissolved in 25 l. 50% aqueous ethanol λ_{max} 236 m μ , $E_{0.2\text{cm}} = 1.5$, — — —, *p* acetamidophenylglucuronide in water λ_{max} 244 m μ , ϵ_{max} 10,800, — · — · —, *p* aminophenylglucuronide in 0.1N KOH λ_{max} 231 m μ , ϵ_{max} 7700 and λ_{max} 291 m μ , ϵ_{max} 1600, · · · · ·, sum of the absorptions of *p* acetamido and *p* aminophenylglucuronides, — · — · —, azoxybenzene in ethanol λ_{max} 235 m μ , ϵ_{max} 9000, λ_{max} 249 m μ , ϵ_{max} 8900, λ_{max} 324 m μ , ϵ_{max} 15,200.

The gum solution showed a band at λ_{max} 236 m μ and an inflexion at 285 m μ . There was no significant absorption at 324 m μ , in which region azoxybenzene absorbs strongly. It can, therefore, be concluded that the gum contains no azoxybenzene, and consequently that phenylhydroxylamine is unlikely to be present (cf. the cases of sulphanilamide and 4,4'-disulphonamidazoxybenzene studied by Williams (1946) and of 4-hydroxylamino-2,6-dinitrotoluene and 2,2',6,6'-tetramino-4,4'-azoxytoluene in TNT metabolism studied by Channon, Mills & Williams (1944)). The peak at 236 m μ may be due to *p* amino and *p* acetamido phenylglucuronide, for if the curves for these compounds are summated, the resultant curve has the same shape as that of the gum. This argument is only valid if irrelevant absorption at the shorter wavelengths is assumed to be negligible. The glucuronide gum may contain, on these grounds, *p* amino and *p* acetamido phenylglucuronide in roughly equal amounts.

RESULTS

The results of the work on isolation of aniline metabolites are summarized in Table 2.

DISCUSSION

The present work shows that the metabolism of aniline in the rabbit is a more complicated process than one would have imagined at first sight and, although a number of the metabolites have been identified, what we think is a major metabolite has,

Table 2 *Derivatives isolated from the urine of rabbits receiving aniline orally*

Derivative	M p	$[\alpha]_D$	Yield as % of dose
(a) Unconjugated			
Aniline hydrogen oxalate	158°	—	6-9
Benzanilide	160°	—	3-9
(b) From glucuronide fraction			
<i>p</i> Toluidine complex of ammonium glucuronate	125-128°	+21° (in 0.4N HCl)	21
<i>p</i> Aminophenylglucuronide	215-216°	-83.4° (in 0.5N H ₂ SO ₄)	0.8
Benzylamine salt of <i>p</i> acetamidophenylglucuronide	195-198°	-59° (in water)	0.5
<i>p</i> Acetamidophenyltriacetylglucuronide methyl ester	205° and 100°	-22.4° (in chloroform)	1
<i>p</i> Aminophenol (by hydrolysis)	182-184°	—	5
(c) From ethereal sulphate by hydrolysis			
<i>p</i> Aminophenol	183°	—	3
<i>o</i> Aminophenol dibenzoate	176-179°	—	0.3
4 Aminoresorcinol (detected)	—	—	?

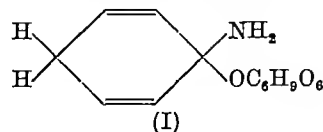
so far, eluded our efforts to isolate and identify it. In what follows we shall attempt to assess, in various ways, the amounts of each metabolite excreted.

The isolation experiments account for nearly 40% of the aniline fed (see Table 2). This figure is made up as follows: free and acetylated *p*-aminophenylglucuronide, 5% (based on the isolation of *p*-aminophenol from the glucuronide gum), free aniline, up to 9% (see § 3 (b)), *p*-aminophenylsulphuric acid, 31%, *o*-aminophenylsulphuric acid, 0.7% (from hydrolysis of the ethereal sulphate fraction, see §§ 4 (a) and (b)). Assuming that each molecule of glucuronic acid isolated as the *p*-toluidine complex is derived from a labile glucuronide generated from one molecule of aniline, then 21% of the aniline fed is excreted as a labile glucuronide (§ 5 (f)).

The excreted free diazotizable amino groups correspond to 40% of the aniline fed (Table 1). The compounds which could contribute to this figure are free aniline (6.5%, see § 3 (b)), *p*-aminophenylglucuronide (5-8%, § 5 (d) and (g)) and *p*-aminophenylsulphuric acid (c. 28%). *o*-Aminophenylsulphuric acid probably does not contribute because Burkhardt & Wood (1929) have shown that when diazotized it loses its sulphate group yielding *o*-aminophenol.

If it is assumed that one molecule of aniline gives rise to one of glucuronide, then 70% of the amine is excreted as conjugated glucuronic acids (Table 1). The isolation experiments indicate that aniline urine may contain three glucuronides, i.e. *p*-amino and *p*-acetamido-phenylglucuronides and a 'labile glucuronide'. The amount of the first two was assessed at 10-15% and, therefore, about 55-60% of the aniline fed may be excreted as the labile glucuronide. We can do little more than speculate at present on the nature of the labile glucuronide.

Aniline urine is reducing and free glucuronic acid in large amounts can be isolated from it as the *p*-toluidine-ammonium glucuronate complex. Now this free glucuronic acid could occur in the urine as such or be produced by the breakdown of a labile glucuronide. The excretion of free glucuronic acid as such, appears to us, at the moment, to be unlikely though not impossible. The existence of a labile glucuronide, however, is feasible on the following grounds. Less than 50% of the aniline fed can be accounted for as diazotizable compounds, the rest may be changed in the body to a non-diazotizable form which is the aglycone of a labile glucuronide. A dihydrohydroxyaniline glucuronide (I) could account for some of the facts. Such a structure as (I) contains an aliphatic amino group, a new asymmetric carbon atom—which could account for the low optical rotation of the urine—and an aldehyde ammonia group—which would make it labile and release ammonia and glucuronic acid to form the *p*-toluidine ammonium glucuronate complex. When this complex is formed from the acidic glucuronide gum no external ammonia is necessary, yet when it is formed from pure glucuronic acid ammonia must be added. Structure (I) is that of a hexa-1,4-diene.



and therefore should show little or no light absorption. It will be recalled that ultraviolet absorption spectrum of the glucuronide gum appears to be that of glucuronides of *p*-acetamido and *p*-amino-phenol only.

We have also proved that aniline is slightly acetylated *in vivo* by the isolation of *p*-acetamido-phenylglucuronide, but no acetanilide was detected. It is possible that here aniline is first oxidized to

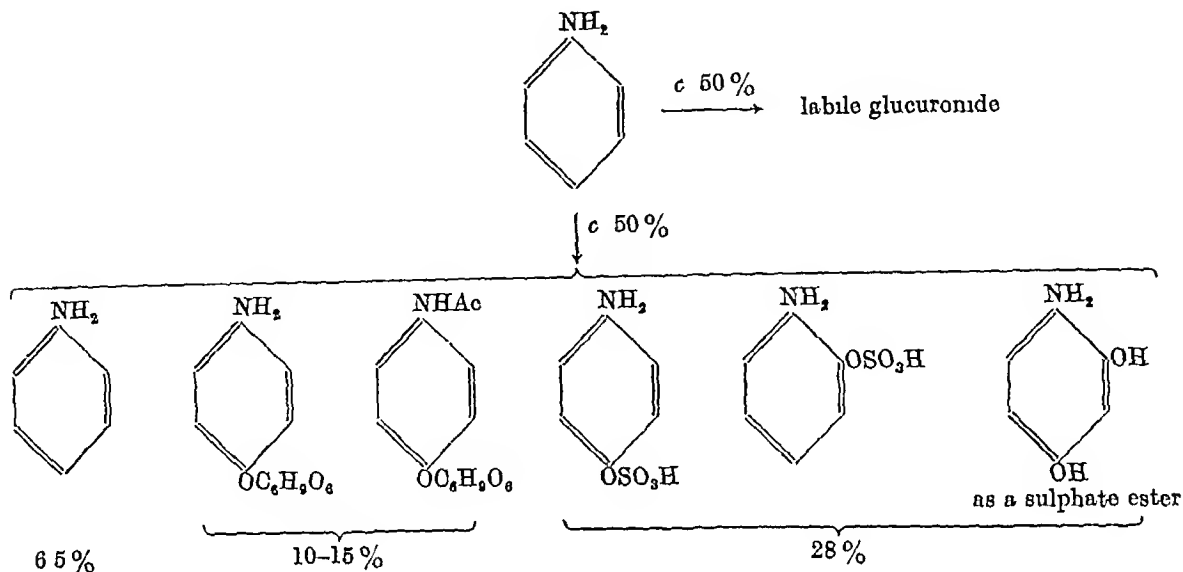
SUMMARY

p aminophenol which is then acetylated and *O*-conjugated, there may be no direct acetylation of aniline

It is clear that the metabolism of aniline is very complex. On available evidence we suggest the following tentative scheme

1 The metabolic fate of aniline in the rabbit has been studied

2 About 28 % of the dose is excreted as the ethereal sulphates of *o*- and *p* aminophenol and



Three aminophenols were found in the urine. The qualitative experiments on the ethereal sulphate fraction suggest that *p* aminophenol preponderates. *o*-Aminophenol and 4-aminoresorcinol were found only in the ethereal sulphate fraction. The only phenol isolated from the glucuronide fraction was *p*-aminophenol, but we cannot eliminate the possibility that the *o* compound is also present in this fraction, for Hanson *et al* (1944) have shown that *o*-aminophenylglucuronide is very resistant to acid hydrolysis. We feel, however, that the *o* glucuronide is not present in appreciable amounts because although it is more easy to isolate from urine (Williams, 1943) than its *p*-isomer, we isolated only the latter from the glucuronide gum.

The detection of 4-aminoresorcinol shows that the *o*- and *p*-aminophenols derived from aniline undergo further oxidation to a trisubstituted benzene. Porteous & Williams (1949) have already shown that benzene gives rise to a trisubstituted compound, hydroxyquinol, in the rabbit.

We suggested in an earlier paper (Smith & Williams, 1948a) that biological oxidation of an aromatic ring takes place at those carbon atoms which possess a certain minimum of electronic activation. On this basis aniline should be oxidized *in vivo* in the *o* and *p*-positions and this is in agreement with our findings (cf acetanilide, Smith & Williams, 1948a).

4-aminoresorcinol. The *o*- and *p* aminophenols were isolated.

3 About 70 % of the aniline fed is excreted as glucuronides, two of which were isolated and identified as *p*-acetamido- and *p*-amino-phenylglucuronide. These two account for 10-15 % of the aniline fed.

4 It is suggested that the main metabolite of aniline is a labile glucuronide. This substance accounts for more than 50 % of the aniline fed and may be a reduced aniline derivative. It also accounts for the reducing properties of aniline urine.

5 The labile glucuronide readily breaks up to give free glucuronic acid, which has been isolated as a crystalline *p*-toluidine ammonium glucuronate complex.

6 No evidence was found to support the view that aniline is converted to phenylhydroxylamine *in vivo*.

7 Aniline gives rise to excretion of small amounts of *N*-acetyl derivatives. Whether or not aniline is directly acetylated has not been proved.

8 Aniline is oxidized in the *o*- and *p*-positions and the significance of this finding is discussed.

9 The metabolites of aniline in the rabbit are not the same as those of acetanilide. The significance of this is discussed.

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Studies in Detoxication

24 THE METABOLISM OF *p* PHENETIDINE (*p* ETHOXYANILINE) WITH SOME OBSERVATIONS ON THE ANISIDINES (METHOXYANILINES)

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This study of the fate of alkoxyanilines in the rabbit was pursued because we wanted to know the effect of alkoxy groups on the biological acetylation of the amino group in compounds of the type $R \cdot C_6H_4NH_2$. The study became of further interest when it was found that administration of *p*-phenetidine and the *o*, *m* and *p* anisidines resulted in the excretion of reducing urines as in the case of aniline (Smith & Williams, 1949b).

Little previous work has been done on *p* phenetidine. Edlefson (1900) showed that, in man, it gave rise to conjugated *p* aminophenol and Elson, Goulden & Warren (1946) suggested that it behaved similarly in the rat.

EXPERIMENTAL

Methods The quantitative estimations of the excretion of glucuronic acid, ethereal sulphate, and diazotizable amino groups in the urine of rabbits receiving *p* phenetidine hydrochloride orally are quoted in the preceding paper (Smith & Williams, 1949b).

Phenetidine was fed as the hydrochloride, m p 234°

The isolation of phenetidine metabolites(1) *The nature of phenetidine urine*

After feeding phenetidine in doses of 0.2–0.5 g/kg, rabbits excreted a brown urine (pH c 8) similar in appearance to aniline urine. It reduced Benedict's reagent and gave a very rapid naphthoresorcinol reaction. It gave a permanganate colour with $FeCl_3$ and a positive diazo reaction.

The urine was optically active. The urine from two rabbits, each of which had received 2 g of phenetidine

hydrochloride, was estimated to contain 4.7 g of extra glucuronic acid. A 10 ml portion of this urine was clarified with dialyzed iron and made up to 20 ml. In a 2 dm tube α_D was -0.42° , whilst normal rabbit urine treated in the same way has no detectable rotation. If the glucuronide in the urine were that of either *p* aminophenol or *p* acetamidophenol, the expected α_D would be c -1.3° . Thus it follows that the glucuronides from phenetidine cannot be entirely *p* aminophenol derivatives.

(2) *Isolation of unchanged phenetidine*

The filtrate from the preparation of the basic lead acetate fraction of phenetidine urine (see p 251) was made alkaline with KOH, filtered and continuously extracted with ether for 4 hr. The bases in the ether were extracted with 2N HCl. This solution was made alkaline and extracted in a funnel with ether. This ethereal solution was dried over NaOH for 24 hr, filtered, and saturated with dry HCl gas. The crystalline precipitate of phenetidine hydrochloride (m p and mixed m p 234°) was filtered off. The yields of unchanged phenetidine in these experiments were 1.5% of the dose at a level of 0.5 g/kg and 2 and 5% at 0.8 g/kg.

(3) *The ethereal sulphate fraction*

(a) *Isolation of 2 hydroxy-4-ethoxyaniline (hydroxyphenetidine)* The 24 hr urine (1250 ml) after feeding 18 g phenetidine hydrochloride was concentrated at 40° *in vacuo* to 250 ml, acidified with dilute HCl, saturated with $(NH_4)_2SO_4$ and extracted with 2 × 250 ml acetone. The acetone extract was made alkaline with solid K_2CO_3 , concentrated *in vacuo* to 100 ml, treated with 1 l dry acetone, filtered and concentrated to 50 ml. The concentrate was now extracted five times with an equal volume of ether to remove free phenetidine (recovered as the hydrochloride, m p 230°) (Found C, 55.1, H, 6.9, N, 7.6. Calc for

$C_8H_{11}ON \cdot HCl$ C, 55.3, H, 7.0, N, 8.0%) Further concentration of the extracted solution caused the separation of urea and some inorganic material which were removed by shaking with 95% ethanol and filtering. The filtrate, now containing largely ethereal sulphates with some glucuronide, was treated with 300 ml absolute ethanol which threw out the glucuronide as a non-reducing flocculent precipitate (600 mg) which we were unable to identify. The ethanol solution was now reduced *in vacuo* to a syrup (30 ml), which at this stage gave negative tests for glucuronic acid, but positive ethereal sulphate and diazo tests. However, we were unable to isolate the sulphate or a derivative in crystalline form. Therefore 10 ml of the syrup were dried *in vacuo*, and the residue boiled for 5 min with 5 ml conc HCl and 20 ml of 5% aqueous $BaCl_2$. After cooling, the $BaSO_4$ was filtered off and the filtrate treated with a little $Na_2S_2O_4$ which removed some of the dark brown colour. Then it was made alkaline with K_2CO_3 , stirred with 5 ml acetic anhydride for 10 min, and extracted with 20 ml chloroform. The extract was dried over $CaCl_2$ and allowed to evaporate at room temperature. A sticky mass of crystals separated which became readily filterable on addition of ether. Recrystallization from benzene gave rectangular plates (170 mg, 2.5% of the dose) of 2-acetoxy-4-ethoxyacetanilide, m.p. and mixed m.p. 127–129° (Found OC_2H_5 , 19.8. Calc for $C_{12}H_{15}O_4N$ OC_2H_5 , 19.0%).

(b) *Synthesis of 2-acetoxy-4-ethoxyacetanilide* 2-Hydroxy-4-ethoxyaniline hydrochloride, prepared according to Henrich & Birken (1913), yielded with aqueous Na_2CO_3 and acetic anhydride 2-acetoxy-4-ethoxyacetanilide, m.p. 127–129° after recrystallization from benzene.

A more convenient method of synthesis starts from 4-nitrosoresorcinol 1-ethyl ether, which was prepared according to the method of Henrich & Rhodus (1902) for the methyl ether. 4-Nitrosoresorcinol 1-ethyl ether (0.5 g) was reduced with 2 g of Sn in 5 ml conc HCl. The reduction mixture was evaporated on the water bath to remove excess HCl, neutralized with $NaHCO_3$ and extracted with ether to remove 2-hydroxy-4-ethoxyaniline. The extract was treated with an excess (10 ml) of acetic anhydride and evaporated *in vacuo* to a small volume. The residue was mixed with an excess of Na_2CO_3 solution, and the whole shaken until the oil, which had separated, crystallized. The 2-acetoxy-4-ethoxyacetanilide (0.27 g) was dried and recrystallized from benzene. It formed rectangular plates, m.p. 127–129° (Found C, 60.5, H, 6.05, N, 6.1, OC_2H_5 , 19.4. $C_{12}H_{15}O_4N$ requires C, 60.7, H, 6.4, N, 5.9, OC_2H_5 , 19.0%).

(4) The glucuronide fraction

(a) *Detection of a glucuronide of p-aminophenol* The basic lead acetate precipitate of a 24 hr urine (340 ml) from two rabbits, each of which had received 2 g of *p*-phenetidine hydrochloride, was prepared in the usual manner. It was suspended in water and Pb removed with H_2S . The lead-free filtrate was concentrated *in vacuo* to a brownish gum (5 g) which had properties similar to that obtained from aniline urine. Part of this gum (4.6 g) was treated with 150 ml of ethereal diazomethane (from 5 g nitroso-methylurea). The ether was removed and the neutral tarry residue dissolved in a mixture of 20 ml pyridine and 20 ml acetic anhydride. After 18 hr at room temperature the mixture was diluted with 100 ml water and extracted with 3 × 20 ml chloroform. After washing with dilute acid and alkali and drying over $CaCl_2$, the chloroform extract was

evaporated, leaving a partially crystalline gum (0.94 g). This product was dissolved in hot ethanol and on cooling to 0°, 150 mg (1.5% of the dose) of *p*-acetamidophenyltri-acetylglucuronide methyl ester (m.p. and mixed m.p. 205–207°), $[\alpha]_D^{15} - 23.5^\circ$ (c, 6.1 in chloroform) separated (Smith & Williams, 1948, 1949b).

Isolation of this compound showed that the glucuronide fraction contained either *p*-aminophenylglucuronide or its *N*-acetyl derivative or both.

(b) *Isolation of p-acetamidophenylglucuronide* The glucuronide fraction was prepared by lead acetate treatment from the 24 hr urine of 6 rabbits which had collectively received 12 g phenetidine hydrochloride. This fraction was concentrated so that 50 ml of an aqueous syrup was obtained. This was now diluted with 400 ml absolute ethanol which was added with shaking. A gum was thrown out and the ethanol (A) removed by decanting. The gum was dissolved in 10 ml water and on treating this with 5 g *p*-toluidine in 5 ml ethanol and cooling to 0° overnight, 8 g of the *p*-toluidine ammonium glucuronate complex (m.p. 125°) crystallized and was filtered off.

The ethanol solution A was now evaporated to 20 ml and again diluted to 250 ml with ethanol to throw out a further portion of insoluble gum. Again the ethanol was decanted and the process repeated. The insoluble gum obtained yielded a further 1 g of *p*-toluidine ammonium glucuronate complex. The mother liquor, after removal of the insoluble gum, was now taken to dryness *in vacuo*. The residual gum (5 g) gave no diazo reaction until hydrolyzed. 4.7 g of it were dissolved in 50 ml 95% ethanol, and 3 ml benzylamine were added. The mixture was diluted to 500 ml with ethyl acetate. From this solution there was obtained 2 g (6.4% of the dose) of the benzylamine salt of *p*-acetamidophenylglucuronide, m.p. and mixed m.p. 195–198° (after recrystallization from 95% ethanol), $[\alpha]_D^{25} - 59^\circ$ (c, 7.8 in water) (Found N, 6.5. Calc for $C_{21}H_{26}O_8N_2 \cdot H_2O$ N, 6.2%).

(c) *Attempted isolation of p-aminophenylglucuronide* *p*-Aminophenylglucuronide was isolated from aniline urine from the mercuric acetate fraction (see Smith & Williams, 1949b, preceding paper). An attempt was made to isolate it from phenetidine urine by a similar procedure. We thought that it might occur in phenetidine urine in small amounts, but its isolation as such or as a benzylamine salt was not achieved.

(d) *Spectroscopic observations on the glucuronide fraction* As in the case of aniline, the absorption spectrum of the glucuronide fraction of *p*-phenetidine urine can be accounted for by the presence of relatively small amounts of amino-phenylglucuronides. An aqueous solution (400 ml) of the glucuronide fraction, prepared, after feeding 8.75 g phenetidine, as in section 4 (a) above, was estimated (naphthoresorcinol method) to contain 7.1 g of glucuronic acid. It was made faintly alkaline with ammonia, diluted 100 times with water and its ultraviolet absorption measured in a Hilger E3 spectroscope. It had a maximum extinction ($E_{0.1\text{ cm.}}$) at 240 μ of 1.2. The form of the absorption curve closely resembled that of *p*-acetamidophenylglucuronide (Smith & Williams, 1948) and the presence of *p*-aminophenylglucuronide was not indicated. If it be accepted that the absorption was due to *p*-acetamidophenylglucuronide, then it can be calculated from the value of $E_{0.1\text{ cm.}}$ that 4.9 g of it is present, i.e. 2.9 g or 41% of the total glucuronic acid.

(5) *The isolation of free glucuronic acid from the urine of rabbits receiving alkoxyanilines orally*

(a) *p* *Phenetidine* The glucuronide gum from the urine of twelve rabbits which had collectively received 24 g of *p* phenetidine hydrochloride was prepared (see section 4 (a)). From this the *p* toluidine ammonium glucuronate complex was prepared as described in the preceding paper on aniline (Smith & Williams, 1949b). The yield was 34 g or 58% of the dose assuming that one molecule causes the excretion of one of glucuronic acid. It was recrystallized (plates) from hot aqueous ethanol (Found C, 56.55, H, 7.4, N, 10.7, OC_2H_5 , 0, glucuronic acid, 47.6, *p* toluidine, 48, H_2O , 4.5, $\text{C}_{20}\text{H}_{29}\text{O}_6\text{N}_3 \cdot \text{H}_2\text{O}$ requires C, 56.45, H, 7.3, N, 9.9, OC_2H_5 , 0, glucuronic acid, 45.6, *p* toluidine, 50.4, H_2O , 4.2%) It decomposed at 125–128° and showed $[\alpha]_D^{20} - 60^\circ \xrightarrow{24 \text{ hr}} +2.5^\circ$ (constant value) (c, 0.5 in 1.3 ethanol water), $[\alpha]_D^{20} + 18 \rightarrow +11^\circ$ (constant) (c, 5.2 in 0.4N HCl).

The complex was analyzed for *p* toluidine by heating on the water bath for 0.5 hr with 0.5N HCl, cooling, then diazotizing and coupling with naphthylethylenediamine. The diazo colour was measured in a Spekker absorptiometer and compared with a standard calibration curve constructed with pure *p* toluidine hydrochloride.

The water content of the complex could not be determined directly by drying to constant weight because it decomposed with loss of toluidine. An approximate estimate of the water content of the sample analyzed above was obtained using Fischer's reagent (pyridine, I_2 and SO_2 , see Fischer, 1935). As mentioned in the preceding paper, we had reason to believe that this compound formed two hydrates similar to the glucose *p* toluidides of Irvine & Gilmour (1909). Our experiments suggested that the hydrate containing $1.5\text{H}_2\text{O}$ was formed when crystallization was carried out at 0° (see section 6 (b)) and the one containing $1\text{H}_2\text{O}$ was formed at room temperature or higher. When the sample above was recrystallized at 0° it gave C, 55.9, H, 7.05, N, 9.6. $\text{C}_{20}\text{H}_{29}\text{O}_6 \cdot 1.5\text{H}_2\text{O}$ requires C, 55.3, H, 7.4, N, 9.7%.

When the compound was dissolved in cold 20% NaOH it gave off ammonia (proved by aeration into picric acid solution, to give ammonium picrate, m.p. and mixed m.p. 270° (decomp.)). In the Van Slyke apparatus the compound released N_2 in 0.5 hr equivalent to 3.2% N, the first formula quoted above requires 3.3% N as NH_4 .

(b) *o* *Anisidine* *o* Anisidine (3.6 g, b.p. 218–220°) was dissolved in the minimum of dilute HCl and the solution diluted to 40 ml. Two rabbits each received 20 ml of this solution. It had a slight narcotic effect but the animals were easily roused by handling. One rabbit died 5 days later. A reducing urine was excreted even during the first 2 hr after feeding. The 24 hr urine (380 ml) was brown and darkened further on standing. The colour was discharged by $\text{Na}_2\text{S}_2\text{O}_4$. The urine reduced Fehling's solution, gave a strong red diazo reaction and an intense Tollens test. The glucuronide gum (3 g) was prepared as for phenetidine urine and, on treatment with *p* toluidine as before, 1.3 g (equivalent to 14% of the anisidine fed) of the *p* toluidine ammonium glucuronate complex (m.p. 125–128° decomp.) was obtained, $[\alpha]_D^{20} + 18^\circ$ (initial value) (c, 3.9 in 0.4N HCl). Absorption spectrum λ_{max} 234 m μ , ϵ_{max} 16,800 in water (Found C, 56.0, H, 7.3, N, 9.6%).

(c) *p* *Anisidine* Two rabbits were each fed with 2 g of

p anisidine (m.p. 57°) in 20 ml of water containing the minimum amount of dilute HCl. The urine was reducing, and the glucuronide gum (3 g), prepared as before, yielded 1 g (equivalent to 9.5% of the dose) of the *p* toluidine ammonium glucuronate complex (m.p. 125–128° decomp.), $[\alpha]_D^{20} + 20^\circ$ initial (c, 5.4 in 0.4N HCl) (Found C, 56.25, H, 7.1, N, 8.8%).

(d) *m* *Anisidine* In a similar fashion the toluidine complex was obtained after feeding *m* anisidine (b.p. 246–247°/720 mm). It had m.p. 125–128° decomp., $[\alpha]_D^{20} + 19 \rightarrow +15^\circ$ (c, 5.1 in 0.4N HCl) and spectral absorption λ_{max} 234 m μ , ϵ_{max} 16,800 and λ_{max} 285 m μ , ϵ_{max} 2600 in water.

(6) *Synthesis of p toluidine ammonium uronate complexes*

(a) *D* *glucurone* When *p* toluidine in ethanol is added to an aqueous solution of glucurone containing a little ammonia, no *p* toluidine complex separates.

(b) *D* *glucuronic acid* *D* Glucurone (0.5 g) was converted into barium glucuronate by treatment with 1 equiv of 0.3N $\text{Ba}(\text{OH})_2$ at room temperature for 3 hr, 0.5 ml of saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added and the mixture filtered from BaSO_4 . To the filtrate was added 0.6 g *p* toluidine in 5 ml ethanol and then the mixture was diluted to 50 ml with ethanol. After 24 hr the crystals (0.54 g, 45% of theory) of the *p* toluidine ammonium glucuronate complex were filtered off and washed with water and ethanol. It was recrystallized from ethanol water and cooled to 0°. Rectangular plates were obtained, m.p. 125–130° (decomp.), $[\alpha]_D^{20} - 59^\circ \xrightarrow{24 \text{ hr}} +2^\circ$ (constant value) (c, 0.3 in 1.3 ethanol water) (Found C, 55.3, H, 7.4, N, 9.7, $\text{C}_{20}\text{H}_{29}\text{N}_3\text{O}_6 \cdot 1.5\text{H}_2\text{O}$ requires 55.3, H, 7.4, N, 9.7%). Ultraviolet absorption in water λ_{max} 234 m μ , ϵ_{max} 16,800 and λ_{max} 285 m μ , ϵ_{max} 2600. It was insoluble in ethanol and organic solvents and sparingly soluble in water. It reduced Benedict's reagent readily and gave a rapid Tollens test for glucuronic acid. It was identical in all respects with the compound obtained from the alkoxy aniline urines.

The same complex was obtained from crude barium glucuronate prepared by acid hydrolysis of Turkey gum.

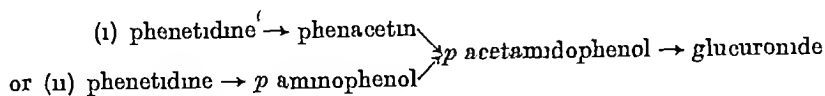
(c) *From (–) Menthyl-D glucuronide* The complex (m.p. 123° decomp.) was obtained in poor yield (7%) from an acid hydrolyzed solution of menthylglucuronide (Found C, 56.3, H, 7.2, N, 9.4% ($[\alpha]_D + 27 \rightarrow +15^\circ$) (c, 2.7 in 0.4N HCl)).

(d) *The complex from D galacturonic acid* When an aqueous solution of *D* galacturonic acid is mixed with an equal volume of an ethanolic solution of *p* toluidine, no apparent change takes place. But as soon as the mixture is treated with a few drops of ammonia solution (sp. gr. 0.88) it crystallizes completely. The *p* toluidine ammonium *D* galacturonate complex, obtained in excellent yield, forms plates, m.p. 135–139° (decomp.) and $[\alpha]_D^{20} - 31 \rightarrow +5^\circ$ (constant value after 24 hr) (c, 1.2 in 50% aqueous ethanol). Its absorption spectrum is similar to that of the glucuronic acid analogue, in water it shows λ_{max} 232 m μ , ϵ_{max} 16,600, λ_{max} 285 m μ , ϵ_{max} 2850 (Found C, 56.5, H, 7.3, N, 9.5, $\text{C}_{20}\text{H}_{29}\text{O}_6\text{N}_3 \cdot \text{H}_2\text{O}$ requires C, 56.45, H, 7.3, N, 9.9%). This was slightly more soluble than the glucuronic acid complex.

DISCUSSION

It is clear that the metabolism of *p*-phenetidine is a complex problem which requires further investigation. So far we have proved that it undergoes (a) de-ethylation, (b) acetylation, and (c) oxidation without de ethylation.

The occurrence of de-ethylation and deacetylation was proved by the isolation of *p*-acetamidophenylglucuronide, which could be formed in two ways as follows



The first of these mechanisms appears to be the most likely, for if *p*-aminophenol were formed we would have expected to isolate its glucuronide, but we did not. For phenetidine the difference between the free and total diazotizable amino groups amounted to 22% (see Table 1 of the preceding paper, Smith & Williams, 1949b). This figure, together with a consideration of the spectrum of the glucuronide gum, suggests that about one quarter of the phenetidine fed is excreted as compounds containing the acetamido group.

It was shown (Smith & Williams, 1949a) that the de ethylation of phenacetin *in vivo* was virtually complete, the main excretion products being *O* conjugates of *p*-acetamidophenol. With phenetidine, however, there is only a partial de ethylation, for we isolated from the urine 2-hydroxy-4-ethoxyaniline in which the original ethoxyl group of phenetidine is intact. It appears, therefore, that breaking of the ether linkage *in vivo* takes place more readily if the *p* amino group is acetylated. The isolation of 2-hydroxy-4-ethoxyaniline is also of interest in relation to the orientation of the hydroxyl group entering the phenetidine molecule. Both the NH_2 and OC_2H_5 groups are *o* *p*-directing (ring activating), but the oxidation takes place *o*- to the amino group. The amino group is known to be chemically more strongly directing than the alkoxyl group (Fieser & Fieser, 1944) and the isolation of 2-hydroxy-4-ethoxyaniline fits in with this view.

Rabbits receiving phenetidine excrete ethereal sulphates corresponding to 30% of the dose. Hydrolysis of the sulphate fraction yielded a reddish tar consisting largely of phenolic substances, from which 2-hydroxy-4-ethoxyaniline was isolated in yields of 8-9% of the ethereal sulphate fraction (i.e. 2.5% of the dose). We failed to detect *p*-aminophenol in this fraction, but there were suggestions that 4-aminoresorcinol was present. It appears, therefore, that the main phenol in the sulphate fraction is 2-hydroxy-4-ethoxyaniline.

The exact nature of the glucuronide fraction of phenetidine urine has not been solved, although it

is clear from this work that it presents, as in the case of aniline, a new aspect of the metabolism of aromatic amines. On measuring the 'extra' glucuronic acid excretion it was found that more than one molecule of glucuronic acid was excreted/molecule of phenetidine fed. The average glucuronide output was 120% of the dose. This high figure could mean that a metabolite carrying two glucuronic acid molecules is excreted. Alternatively, it may mean that phenetidine stimulates the excretion of free glucuronic acid. The *p*-toluidine complex

of glucuronic acid was isolated in large amounts, equivalent to 58% of the phenetidine fed, and we believe that a considerable proportion of phenetidine is excreted as a labile glucuronide. A minor component of the glucuronide fraction is *p*-acetamidophenylglucuronide, which was isolated. Although

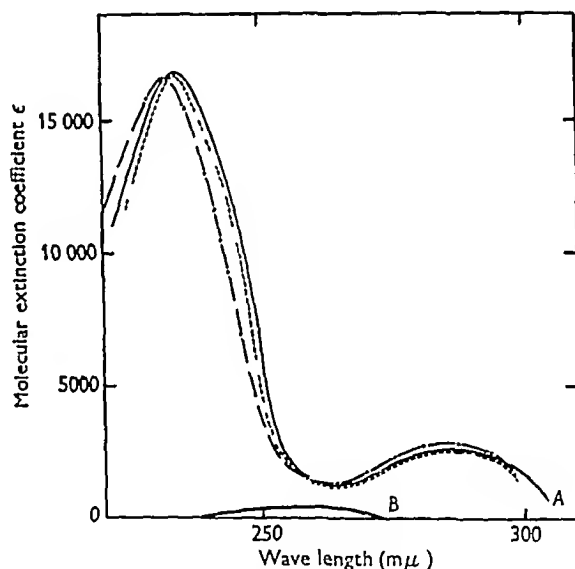
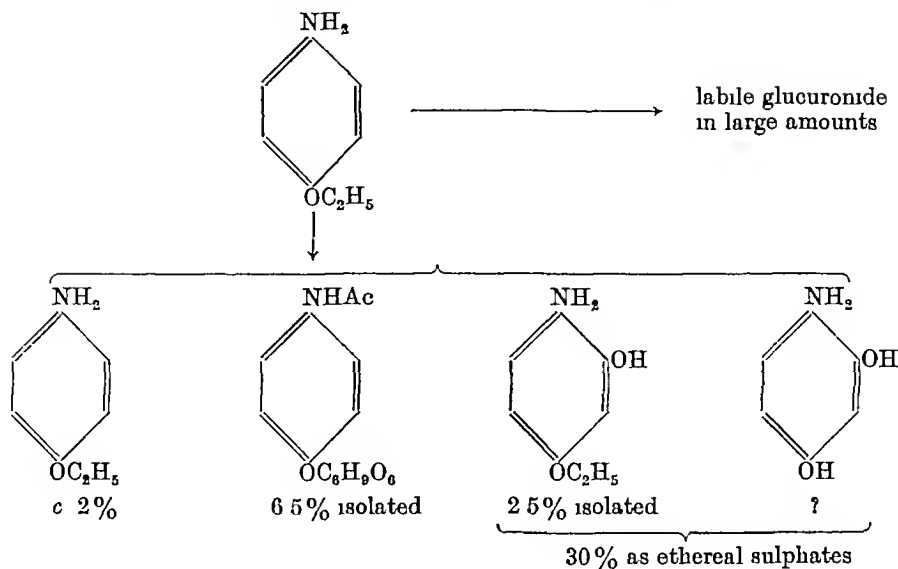


Fig 1 Ultraviolet absorption spectra of *p* toluidine ammonium uronate complexes — A, *p* Toluidine-ammonium D glucuronate complex from aniline urine in water λ_{max} 234 $\text{m}\mu$, ϵ_{max} 16,900 and λ_{max} 285 $\text{m}\mu$, ϵ_{max} 2700, B, same as A but in N HCl λ_{max} 261, ϵ_{max} 400, — — — synthetic *p* toluidine ammonium D glucuronate complex (prepared from D glucuronic acid) in water λ_{max} 234 $\text{m}\mu$, ϵ_{max} 16,800 and λ_{max} 285 $\text{m}\mu$, ϵ_{max} 2600, - - - *p* toluidine ammonium D galacturonate complex (prepared from D galacturonic acid) in water λ_{max} 232 $\text{m}\mu$, ϵ_{max} 16,600 and λ_{max} 285 $\text{m}\mu$, ϵ_{max} 2850

we isolated *p* aminophenylglucuronide from aniline urine, it was not found in phenetidine urine, a fact which is supported by the very low amount (3% of the dose) of free diazotizable amino groups found in phenetidine urine.

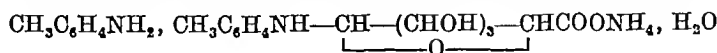


The evidence presented here on the fate of phenetidine in the rabbit does not allow us to make very definite conclusions and we suggest the above scheme very tentatively.

The p-toluidine glucuronate complex and the labile glucuronides derived from aromatic amines The *p*-toluidine ammonium glucuronate complex has been isolated from *p*-phenetidine (58% of the dose), aniline (21%), *o*-anisidine (14%), *m*-anisidine (12%) and *p*-anisidine (9.5%) urines. Only traces of this complex were obtained from acetanilide and phenacetin urines (Smith & Williams, 1949*a*), these traces probably being formed as a result of the production of very small amounts of aniline and phenetidine by deacetylation. It is clear, therefore, that the hypothetical labile glucuronides are only produced from the free aromatic amines and not from the *N*-acetylated amines. Further investigations are in progress on the fate of other types of

acid is derived from labile glucuronides and that the one from *m*-anisidine has some stability.

The structure of the crystalline *p*-toluidine ammonium glucuronate complex also requires discussion. From its absorption spectrum (see Fig. 1) it is clear that the only absorbing component is *p*-toluidine (according to Dede & Rosenberg (1934) *p*-toluidine shows two absorption bands in water, at 234 $\text{m}\mu$, $\log \epsilon$ 3.91 and 288 $\text{m}\mu$, $\log \epsilon$ 3.15). Its rising mutarotation in ethanol-water ($[\alpha]_D -60 \rightarrow +2^\circ$) suggests that it is a *p*-toluidide (i.e. a *N*-glucuronide) of β configuration. On keeping a few weeks the compound turns a light brown. It has no true melting point but decomposes fairly consistently at 125–128°. The analytical data on the compound suggest that it contains 1 molecule of *p*-toluidine, 1 of the *p*-toluidide of ammonium glucuronate and 1 or 1.5 molecules of crystal water. It may be tentatively formulated as



substituted anilines, and so far we have found that *p*-bromoaniline does not give rise in the rabbit to reducing urines. In the case of the naphthylamines, we have found that the 1-derivative gives rise to a urine which reduces Fehling's but not Benedict's reagent, whereas with the 2-derivative the urine only slightly reduced Fehling's solution.

The *p*-toluidine complex cannot be prepared directly from the 'arylamine urine', but is obtained readily from the glucuronide gums prepared at ordinary temperatures by systematic lead acetate precipitations. In the case of *m*-anisidine the complex is only obtained with difficulty from the glucuronide gum and often only after the addition of a little ammonia. The addition of ammonia is unnecessary in the other cases, e.g. aniline and phenetidine. This strongly suggests that the glucuronic

D Galacturonic acid gives the same type of complex whose properties and spectrum (see Fig. 1) are similar to those of the glucuronic acid complex.

SUMMARY

1 The fate of *p*-phenetidine in the rabbit has been studied, and some observations have been made on the *o*-, *m*- and *p*-anisidines.

2 About 30% of the phenetidine fed is excreted as ethereal sulphates. The main phenol in the sulphate fraction is 2-hydroxy-4-ethoxyaniline which was characterized as 2-acetoxy-4-ethoxyacetanilide, the synthesis of which is described for the first time. It is suggested that 4-aminoresorcinol is also present.

3 A considerable amount of glucuronide is excreted which appears to be largely a labile glucuronide, the nature of which has not been elucidated.

4 *p*-Acetamidophenylglucuronide has also been isolated from the urine. This substance was obtained in 6% yields, but may occur in the urine to the extent of 20% or more.

5 A *p*-toluidine-ammonium glucuronate complex has been isolated from *p*-phenetidine, *o*, *m*- and *p*-anisidine urines. The yield from phenetidine urine was high.

6 The *p*-toluidine complexes of ammonium glucuronate and galacturonate have been synthesized and studied, and suggestions have been made regarding their nature.

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A Method for Continuous Graphic Recording of Radioactive Tracer Concentrations from Various Body Regions Simultaneously

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In measuring the changing content of radioactive tracer in different organs of man and animal, it is of great interest to investigate the fate of the tracer at very short time intervals after administration, and also to measure its accumulation simultaneously in several parts of the body. Since information of this kind may not easily be obtained by use of the usual dynatron scalers, certain modifications were necessary to allow continuous recording from different scalers simultaneously.

EXPERIMENTAL

Apparatus

The internal wiring of the dynatron scaler was slightly modified. The connexions to the internal Post Office meter were diverted to a double pole, double throw, changeover switch (*CH*, Fig. 2), thus enabling the operator to switch over to an external 2 pin socket (*S*). Socket and changeover switch were both mounted on the front panel of the scaler. The 2 pin socket facilitated the connexion with an external Post Office meter (*M*), which had the same resistance (2300 Ω) as the meter mounted inside the scaler. It was possible, by these means, to record every tenth or hundredth impulse on either the internal or external Post Office meter. The latter (Fig. 1, and *M*, Fig. 2) was fitted with a small clamping block (*CB*, Fig. 1) on the moving armature, and in this the stem of the writing point (*WP*) was clamped. A brass rod (*R*) was screwed into the back of the meter, permitting the meter to be mounted on a kymograph, where the impulses were continuously recorded.

The Geiger Müller counters consisted of tungsten helix γ tubes, made by 20th Century Electronics Co. Ltd. They were shielded with lead tubes in which a $\frac{1}{4}$ in. wide longitudinal slot had been made and the tubes clamped in such a way that the slot was placed just over the organ under investigation. The counters were further shielded with black paper, which nullified the photosensitivity of the tubes. An example of the arrangement of the counters, as used in clinical investigations, is illustrated in Fig. 3.

For recording the impulses coming from different Geiger Müller tubes located on various parts of the body, different scalers were employed, each connected with its external counter writer, thus permitting simultaneous recording of fluctuations of tracer concentrations.

Clinical investigations

The assembly described above has been so far used clinically for recording changes in ^{131}I concentration only. Prior to the injection of ^{131}I the kymograph was started and the background counts recorded. Then, without stopping the recording mechanism, a dose of ^{131}I was given.

Animal experiments

The method has proved particularly valuable in recording changes of blood ^{131}I concentrations.

A length of antioxidant-free 'Telcothene' tubing (obtained from Telegraph Construction and Maintenance Co. Ltd.) of 1.5 mm. bore was wound round a γ counter tube in the form of a spiral, which was secured by sticking over with transparent adhesive tape. The total volume of this spiral was approximately 4 ml. (see Fig. 4).

In anaesthetized and heparinized rabbits the carotid was exposed, the blood flow interrupted, and cannulae (*C1* and

C2) were inserted in the proximal (CPR) and distal (CD) parts, as shown in Fig 4. The proximal cannula was first connected with the spiral (TS) and the blood allowed to run through until the spiral was full. The free end of the spiral was then connected to the distal cannula avoiding the entry of air bubbles into the system. The T-pieces (T1 and T2) permitted the taking of samples, the control of flow and, if required, the measurement of blood pressure. The same counter, once prepared, was used for several control experiments. After each experiment, the spiral was cleaned with

The results of a typical animal experiment showing the changes of ^{131}I in the blood stream and thyroid of a rabbit are illustrated in Fig 5. It will be noted that about 60 sec elapse before complete distribution takes place in the blood, and that the concentration rapidly falls to 50% of the maximum value within the first 240 sec. Further, it shows how readily the thyroid takes up iodine, which reaches a high level during the first 30 sec, and

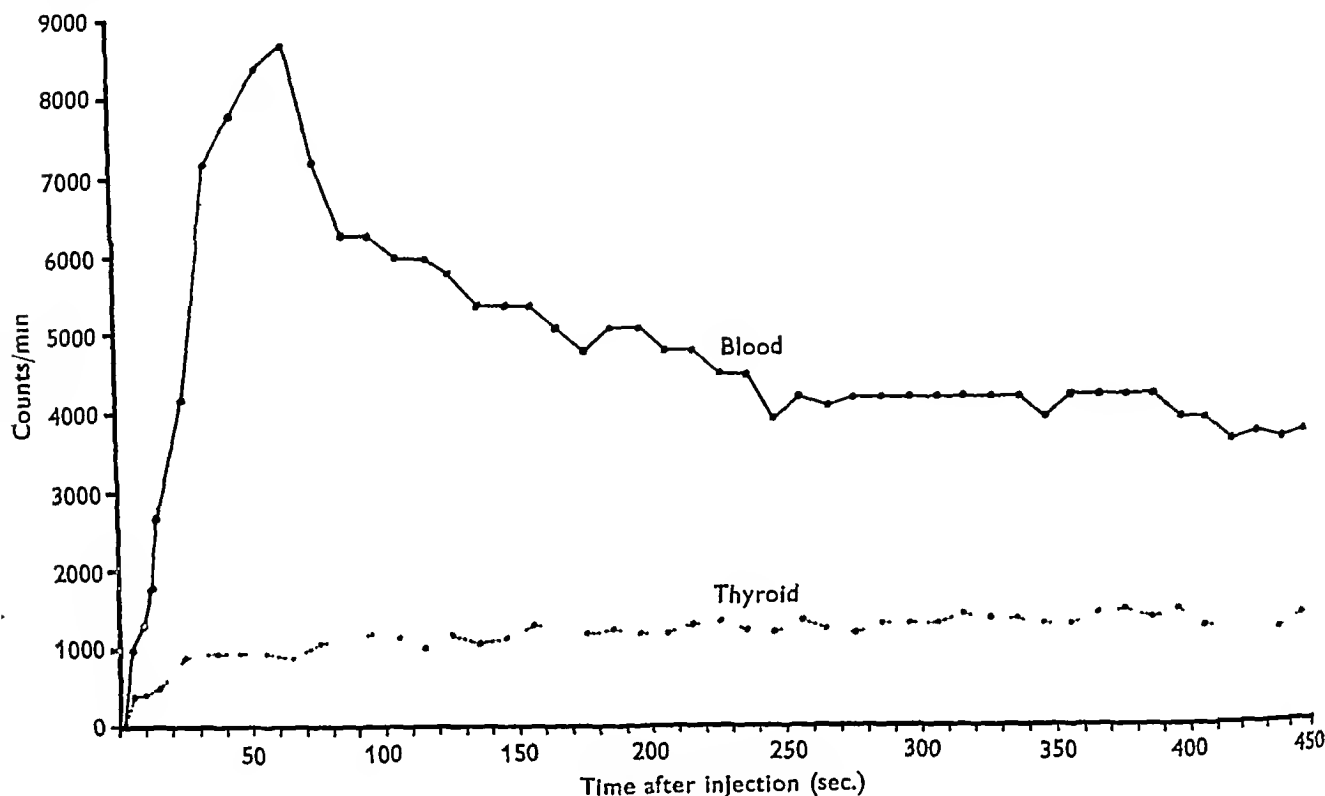


Fig 5

out detachment from the counter tube. After removal from the animal, water was drawn through the spiral by means of a filter pump, followed by washing through with detergent solution, it was finally rinsed again with water and dried.

After the operative procedures were completed, and the background count recorded, ^{131}I was injected intravenously. The fate of the tracer substance was then recorded from the moment of its entry into the blood stream. The number of impulses could be assessed, for instance, at intervals of 10 sec. In the experiments with which we were concerned, the ^{131}I uptake of the thyroid was measured at the same time by a GM 4 counter enclosed in a lead tube (see Fig 4).

RESULTS

Very soon after injection of ^{131}I into a human subject the thyroid counter began to show an increased activity, whilst changes in the bladder readings occurred later. The gradient of ^{131}I uptake by the thyroid and other organs was found to vary considerably in different patients. Clinical diagnostic results achieved with this method will be reported later.

then increases more slowly over the following 420 sec.

The method appears to have considerable heuristic value, and is now used in these laboratories for investigations relating to iodine metabolism.

SUMMARY

1 A polygraphic device for recording continuous and simultaneous changes of the radioactive tracer concentrations in different body regions is described.

2 Examples of the applicability of the method are described.

3 It was possible, by the use of this method, to study the fate of ^{131}I from the moment of its entry in the blood stream.

The authors wish to express their thanks to the Medical Research Council for supplies of radioactive material, to Mr H A Adams (Chief Engineer of Bristol Mental Hospitals) for his valuable help and co operation, and to Dr J B Brerley (of the Anatomy Department, University of Bristol) for preparing the diagrams (Figs 1 and 4).

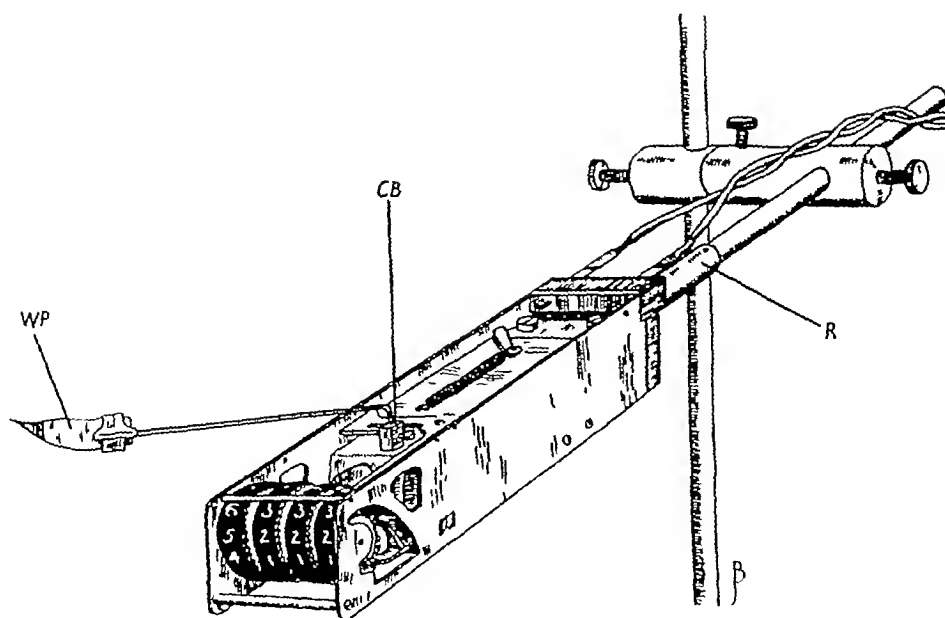


Fig 1 The counter and writer

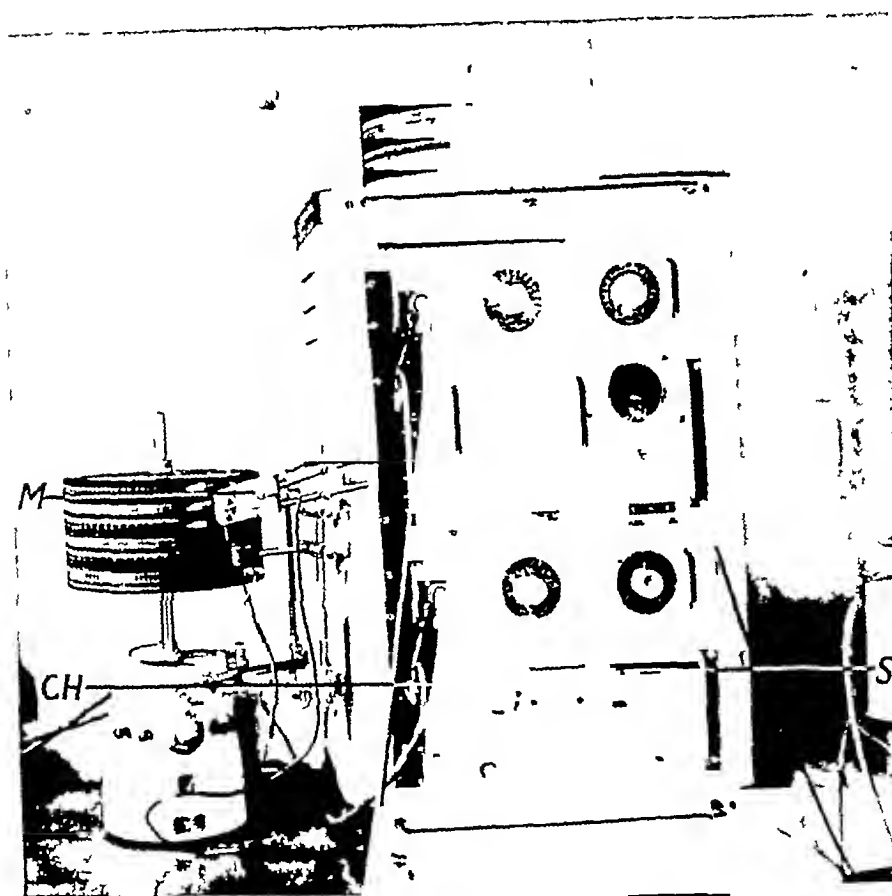


Fig 2 Showing connexion between scaler and writer

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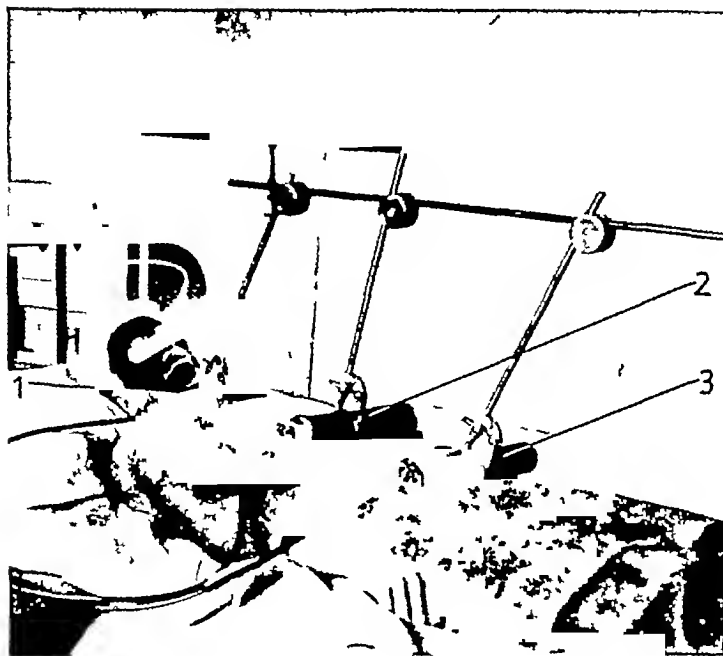


Fig 3 Showing arrangement of counters for simultaneous recording from different parts of the body
1, thyroid region, 2, liver region, 3, bladder region

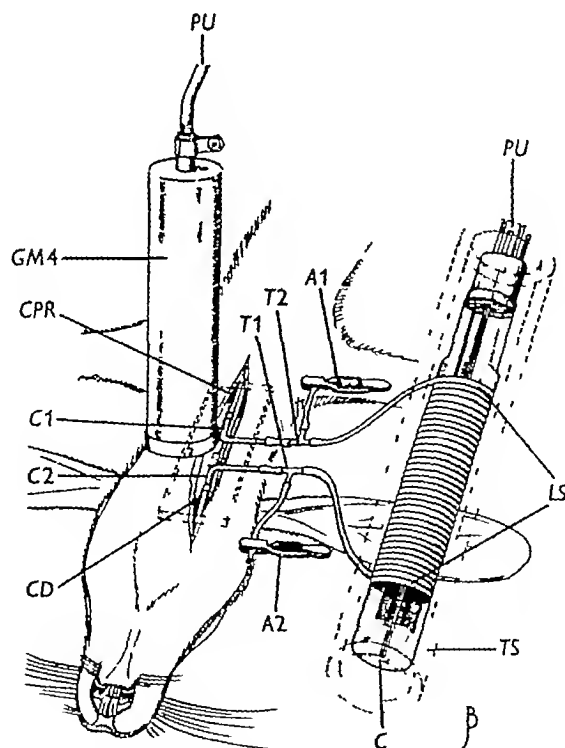


Fig 4 Schematic diagram of the arrangement for simultaneous recording of tracers from blood stream and thyroid PU, leads to probe unit, GM4, GEC Geiger Muller counter inside lead shield, C, 20th Century Electronics counter, tungsten helix, TS, Telcothene spiral, LS, lead shield, CPR, proximal part of carotid, CD, distal part of carotid, C1 and C2, glass cannulae, T1 and T2, glass T-pieces, A1 and A2, artery clamps

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The Influence of the Pituitary on Phosphorus Metabolism of Brain

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A previous investigation (Reiss & Rees, 1947) dealt with the carbohydrate metabolism of rat brain under different endocrine conditions. The hexokinase activity of grey matter was found to be increased after hypophysectomy, as was also the anaerobic glycolysis, the latter function was restored to normal by treatment of the animals with preparations of corticotrophic hormone.

On account of the important part played by phosphate in carbohydrate metabolism, it was considered of interest to follow these experiments with investigations of the phosphorus metabolism, and the present paper describes some investigations which have been made in this field by radioactive tracer methods.

Previous observations of the uptake of radioactive phosphorus by brain after injection have been reported. Borell & Örström (1947) demonstrated by this means that the phosphate turnover in the pineal body is considerably higher than for any other part of the brain. Similar experiments, carried out in animals of different ages, were recorded by Friess & Chaikoff (1941) and by Schachner, Friess & Chaikoff (1942), who also examined the distribution of absorbed phosphorus of fractions of different solubility, especially the lipid fraction.

The experiments described in this communication were designed mainly to reveal differences in behaviour between the brains of normal and hypophysectomized animals.

METHODS

Measurements Measurements of radioactivity on dry samples were made with G M 4 Geiger counter tubes (General Electric Co Ltd). All samples were placed in the lead castle at the same distance from the counter. Liquid samples were dealt with by the use of the Alltools M R C 1 liquid counter.

Animals Rats (both hooded and Wistar types) were used throughout the investigations, and were killed by decapitation. Generally pairs of rats, as nearly as possible equal in weight, were used, one serving as control to the treated animal. Where bigger groups of animals were used, one third to one half of the total number were controls. Hypophysectomy was performed by the paratracheal route, and rats were used at varying intervals after operation.

Preparation of material for measurements Small amounts of tissue were best dealt with by mounting on filter papers of 2.5 cm diam which had been previously weighed on a torsion balance. After spreading the tissue on the paper, the combined weight was read and the material dried in an oven at 100°. Very thin layers were thereby obtained, and the weights of samples thus used could be as low as 1.0–2.5 mg for a whole pineal. For other organs, such as brain, liver, or spleen, the weights were within the range of 20–60 mg. Blood (20–40 mg) was soaked into the paper, and, after first drying in air, was dried in the oven as before. When ready the papers were laid flat on a sample tray in the lead castle containing the G M 4 counter. Repeat counts for the same sample varied by 1–5%. In some experiments the β radiation of the brain surface only was measured. The brain (without olfactory lobes) of the decapitated animal was prepared, and, after removal of traces of blood, was placed on a microscope slide in the centre of the lead castle. The counts/min for upper and lower surface are combined in the tables. For liquid counts the whole brain (excluding olfactory lobes) was first homogenized in the presence of a few ml 25% trichloroacetic acid with an Elvehjem homogenizer (Potter & Elvehjem, 1936). After centrifuging, the supernatant fluid was made up to 10 ml with water, and the solution was thoroughly mixed and immediately poured into the liquid counter for measurement. Lipid material was then extracted from the precipitate by suspending in Bloor's mixture. The mixture was placed in a hot water bath until the solvent was brought to the boil, then cooled, the volume made up to 50 ml with fresh Bloor's solvent, and the mixture centrifuged. Samples of 10 ml were taken for measurements.

The residue finally remaining from the acid and lipid extractions was ashed by heating in a hard glass tube over a microburner with 2 ml 60% HClO_4 , until the charred material had clarified and the mixture become homogeneous. After cooling, this was made up to 10 ml with water and transferred to the liquid counter.

RESULTS

Representative results of a series of twenty one experiments in which the animals were sacrificed 40 min after intravenous injection with ^{32}P , and the ^{32}P uptake by the various organs measured, are indicated in Table 1. It will be seen that the phosphorus uptake of grey matter in the normal animal is very low, being only about one eighth of that of the liver. The rate of uptake by the pineal is considerably higher than for other organs investigated, the pineal accumulates (per 100 mg fresh

Table 1 *Comparison between ^{32}P uptake of normal and hypophysectomized animals and hypophysectomized animals injected with corticotrophic hormone*

(Intravenous injection of $2\mu\text{C}$ ^{32}P 40 min before dissection)

Organ	Counts/min /100 mg tissue		
	Normal animals	Hypophysectomized animals	Hypophysectomized animals injected with corticotrophic hormone 1 hr before ^{32}P
Brain, grey matter	35, 48, 60	123, 162, 140	34, 72
Olfactory lobe	118, 93, 88	33, 64, 76	49, 53
Pineal	381, 333, 280, 216	1028, 532, 644, 728	293, 272
Liver	228, 196	259, 320	296, 280
Adrenal	64, 78	85, 96	92, 96
Thymus	109, 145	50, 76	90, 63
Testis	89, 94, 82	49, 56, 62	66, 55, 48
Blood	21, 51, 23, 28, 42	24, 42, 17, 38, 48	18, 12, 16

Table 2 *Comparison between ^{131}I uptake of normal and hypophysectomized animals*

(Intravenous injection of $2\mu\text{C}$ ^{131}I 1 hr before dissection)

Organ	Counts/min /100 mg tissue	
	Normal animals	Hypophysectomized animals
Brain, grey matter	129, 172, 145, 110, 160	138, 123, 170, 165, 142
Olfactory lobe	185, 275, 298, 242, 152	236, 270, 210, 190, 196
Pineal	480, 606, 820, 704, 634	940, 1260, 1285, 1122, 1420
Liver	328, 382, 422	349, 304, 318
Adrenal	264, 325, 364, 411, 321	450, 443, 360, 520, 288
Thymus	344, 343, 480	483, 354, 512
Testis	342, 556, 193, 664	336, 321, 282, 312
Thyroid	4850, 6148, 7261, 5224, 5368	620, 678, 1500, 996, 352, 826
Blood	892, 680, 630, 721, 866	531, 842, 1240, 1260, 482, 636

tissue) about ten times as much phosphorus as brain, six times as much as the adrenal and 50 % more than the liver

Hypophysectomy appears to bring about a general increase in the absorption. In different experiments the increase in the case of brain amounted to 50–500 %. The pineal shows an increase in uptake of 70–900 %, while the liver showed increases of 0–100 %

The effect of corticotrophic hormone is clearly demonstrated. Administration to the hypophysectomized animal of about 3 sudanophobic units 1 hr before injection of ^{32}P reduced the phosphorus uptake of the brain and of the pineal to a normal level

That the high activity of the pineal is not restricted to phosphorus only is shown in Table 2, which gives the results of parallel experiments carried out with ^{131}I (the only other radioactive indicator available at these laboratories). After intravenous injection, more ^{131}I is taken up by the pineal than by any other tissue with the exception of the thyroid. The iodine uptake by the pineal after hypophysectomy is increased in the same

manner as the phosphorus uptake, but no corresponding behaviour was shown by brain

Table 3 illustrates experiments where radiation of the brain surface was measured at different intervals after injection of various doses of ^{32}P . There is, without exception, a considerable increase in phosphorus uptake ranging between 53 and 588 %, and this increase is borne out by the results of measurements on the trichloroacetic acid extract of the whole brain. At the same time, the content of ^{32}P in the lipid phosphorus fraction showed little change when measured 1 hr after injection of the phosphorus. There was, however, a considerable increase, compared with normal control animals, 17.5 hr after injection

DISCUSSION

The increase of phosphorus uptake of the brain after hypophysectomy should be considered in combination with the previous finding of Reiss & Rees (1947) that hypophysectomy results in increased hexokinase activity and anaerobic glycolysis

The increased activity of the brain with regard to

Table 3 Investigation of the whole brain after injection of ³²P

Dose of ³² P injected (μc)	Time interval between injection and dissection (hr)	Normal animals (counts/mm)				Hypophysectomized animals (counts/mm)				Percentage increase in ³² P uptake after hypophysectomy			
		Brain surface	10 ml trichloro acetic acid extract of total brain	10 ml Blood extract of total brain	10 ml trichloro acetic acid extract of total brain	Brain surface	10 ml trichloro acetic acid extract of total brain	10 ml Blood extract of total brain	Brain surface	Trichloro-acetic acid extract	Blood extract	Brain surface	Trichloro-acetic acid extract
7	72	347	—	—	—	657	—	—	89	—	—	—	—
7	96	264	—	—	—	470	—	—	78	—	—	—	—
5	4	208	—	—	—	318	—	—	53	—	—	—	—
3	4	140	—	—	—	232	—	—	66	—	—	—	—
2	24	24	18	—	—	123	101	—	430	960	—	—	—
2	2	22	27	—	—	151	224	—	588	730	—	—	—
2	2	89	98	—	—	263	425	—	191	338	—	—	—
2	1	40	66	—	—	93	152	—	132	283	—	—	—
2	0.5	67	39	—	—	132	145	—	97	273	—	—	—
6	16	77	86	—	—	142	155	—	91	80	—	—	—
6	17.5	—	96	90	—	—	127	145	—	32	61	—	—
2	17.5	—	53	45	—	—	126	150	—	137	233	—	—
2	17.5	—	61	40	—	—	148	135	—	142	234	—	—
2	1	—	67	45	—	—	147	65	—	119	44	—	—
2	1	—	89	0	—	—	95	5	—	7	—	—	—

phosphorus may possibly be due to increased carbohydrate utilization, and this supposition would appear to be justified by the large increase of phosphorus in the acid-soluble fraction, which contains the various carbohydrate phosphoric esters. These observations are interesting in the light of certain long-established therapeutic applications of phosphates: the administration of sodium dihydrogen phosphate or phosphorus-containing syrups in cases of fatigue may be justified on the basis that freshly administered inorganic phosphate can be rapidly mobilized for phosphorylation processes in the brain, thus providing a stimulus to carbohydrate metabolism.

In this connexion there may be some grounds for the belief that a more sensitive β-counter could furnish information of diagnostic importance, by revealing something of the state of carbohydrate metabolism in the human brain, through observations of rates of radioactive phosphorus uptake. It would be wise, however, to suspend judgement on this point, until much more work of a fundamental character is accomplished.

The finding of Borell & Örstrom (1947) that the pineal has the largest phosphorus uptake/100 mg is confirmed. It may be doubted, however, whether their deduction that the pineal plays an important role in phosphorus metabolism is justified, in view of the equally high activity of this gland with regard to ¹³¹I, demonstrated in the present research. No explanation can at this stage be offered for this behaviour, but it may be permissible to offer the suggestion that the pituitary gland can exert some inhibitory function, controlling the activities of the pineal.

SUMMARY

- 1 Previous observations by other workers on the uptake of radioactive phosphorus by the brain after injection into normal rats are confirmed, comparative measurements on other organs have been made.
- 2 The effect of hypophysectomy is to bring about a general increase in the absorption of phosphorus, particularly by the brain and the pineal.
- 3 Administration of corticotrophic hormone to the hypophysectomized animals, before injection of phosphorus, restores the absorption to a normal level.
- 4 It is suggested that the increased rate of ³²P uptake of the brain in the hypophysectomized animals is connected with increased carbohydrate utilization.

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Studies on Cholinesterase

6 THE SELECTIVE INHIBITION OF TRUE CHOLINESTERASE *IN VIVO*

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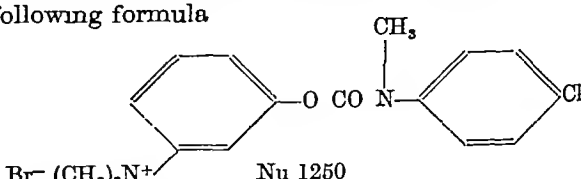
When the distinction was made between two types of cholinesterase (ChE) in the animal body, these enzymes were called true cholinesterase and pseudo-cholinesterase (Mendel & Rudney, 1943*a*). The name pseudo-cholinesterase was chosen because it was deemed unlikely that this enzyme could deal effectively with acetylcholine *in vivo* in view of its relative inactivity towards low concentrations of that substrate. To determine the validity of this assumption Hawkins & Gunter (1946) employed a selective inhibitor of pseudo cholinesterase, the prostigmine analogue Nu 683. *In vivo* experiments with this compound showed that a prolonged and almost complete inhibition of pseudo-cholinesterase in plasma and tissues does not evoke any reaction indicative of the accumulation of acetylcholine. Thus it appeared that in conditions in which the activity of true cholinesterase is relatively unimpaired pseudo cholinesterase plays no essential role in the removal of acetylcholine *in vivo*.

The possibility remained, however, as these workers pointed out, that an auxiliary role might be assumed by pseudo cholinesterase, namely, that of serving as a substitute for true cholinesterase whenever the activity of the latter enzyme is impaired. Proof of this hypothesis has hitherto not been put forward. It has awaited the finding of a compound which not only possesses the property of acting as a selective inhibitor of true cholinesterase, but which can also be administered *in vivo* without eliciting any effects other than those attributable to its cholinesterase inhibiting action.

Selective inhibitors of true cholinesterase have been described previously. Zeller & Bissegger (1943)

have shown that high concentrations of caffeine inhibit the cholinesterases of erythrocytes and brain, two examples of true cholinesterase (Mendel & Rudney, 1943*a, b*), without significantly affecting the cholinesterase activity of human plasma which contains mainly pseudo-cholinesterase (Mendel, Mundell & Rudney, 1943). Recently, Adams & Thompson (1948) have demonstrated that certain 'nitrogen mustards', particularly di-(2-chloroethyl) methylamine (DDM), act as selective inhibitors of true cholinesterase.

The present communication deals with a cholinesterase inhibitor which, apart from possessing a selective inhibitory action on true cholinesterase, produces no effects *in vivo* that could not be attributed to the accumulation of acetylcholine. This compound, a prostigmine analogue, the *N-p* chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250, Aeschlimann & Stempel, 1946), has the following formula



Br⁻ (CH₃)₃N⁺ Nu 1250

The aforementioned properties of Nu-1250 suggested its use as a tool in determining whether pseudo-cholinesterase can assume an auxiliary role in the hydrolysis of acetylcholine when the activity of true cholinesterase is impaired. The results of experiments dealing with this question are reported here.

METHODS

Substrates

Acetylcholine chloride (Ach), Hoffmann-La Roche, Basel, Switzerland Benzoylcholine chloride (Bch), kindly supplied by Hoffmann La Roche, Inc., Nutley, N J Acetyl β methylcholine chloride (Mch), Merck's Mecholyl

In all cases, the final concentration of substrate in the reaction mixture was sufficient to afford complete saturation of the enzyme, viz 0.0012M Ach for true cholinesterase, 0.06M Ach for pseudo cholinesterase, 0.03M Mch and 0.006M Bch for the same two enzymes respectively (Mendel et al 1943)

Estimation of enzyme activity

The activity of the cholinesterases was measured manometrically by Warburg's method at 37.5° in a medium containing 0.025M NaHCO₃ saturated with 5% CO₂ in N₂ (pH 7.4). Blank determinations were run for each substrate to estimate the extent of non enzymic hydrolysis.

The enzyme preparation was placed in the main compartment of the Warburg vessel, the substrate in the side arm, and the inhibitor (0.1 ml.) was added to the enzyme bicarbonate mixture in the main compartment. The total fluid volume was 5 ml. A 15 min period was allowed for the attainment of thermal equilibrium, following which the substrate was tipped in from the side arm. Readings were started 5 min later, and were repeated at appropriate intervals for the next 20 min, or, in cases where low substrate concentrations were used, until the rate of the reaction began to fall.

Source of enzyme

Plasma Freshly drawn, oxalated blood was used. The cells were separated from the plasma by centrifugation. The volume of plasma used (0.1–1.0 ml) depended on the activity, which varied from one species to another. Following the injection of the inhibitor into experimental animals, the activities of the cholinesterases were measured in undiluted plasma to prevent the inhibitor enzyme complex from dissociating upon dilution. This procedure necessitated correction for the retention of CO₂ (Warburg, 1925).

Erythrocytes After removal of the plasma, the cells were washed three times with 0.9% NaCl, and haemolysis was then brought about by the addition of three times their volume of distilled water. 0.2 ml of the haemolysate was used in the estimation of enzyme activity.

Fractionated rat plasma The plasma of six male rats was brought to 0.5 saturation with (NH₄)₂SO₄. After 30 min the mixture was centrifuged and the supernatant fluid (S) decanted. The precipitate was then washed with 0.4 saturated (NH₄)₂SO₄ for 20 min, centrifuged, and the remaining precipitate (P) suspended in a small volume of water. Both S and P were dialyzed against tap water for 18 hr. Using Mch and Bch as substrates, it was found that fraction S contained pseudo cholinesterase but no true cholinesterase, whilst fraction P contained the true cholinesterase, but only a trace of pseudo cholinesterase.

Dog brain The brain was removed as soon as possible after death, and a suspension of the nucleus caudatus was prepared by grinding the tissue in three times its weight of

distilled water. The suspension (0.1 ml) was used in the determination of enzymic activity.

Dog pancreas The gland was obtained from the same animal from which the brain had been taken. The larger blood vessels and fibrous tissue were removed, and a suspension was prepared by grinding the pancreas with nine times its weight of distilled water in a Waring Blendor and subsequently straining it through a fine sieve. The suspension (0.1 ml) was used in the experiments reported.

Inhibitor

A stock solution of Nu 1250 (10⁻³M) in distilled water was prepared every second day. Subsequent dilutions were made from this solution as required. In contrast to the experiences with diisopropyl fluorophosphonate (DFP) and DDM, no significant change in potency was evident within a 36 hr period (Mol wt of Nu-1250 = 3994).

RESULTS

(A) *In vitro experiments*

The following preliminary experiments were performed to assess the suitability of Nu-1250 as a tool for testing whether pseudo-cholinesterase can play an auxiliary role in the hydrolysis of acetylcholine *in vivo*.

(1) In order to find a suitable animal for the proposed *in vivo* experiments, the margin between the sensitivities of true cholinesterase and pseudo cholinesterase in various species towards Nu-1250 was determined.

(2) Since acetyl β methylcholine and benzoylcholine were to be used as substitutes for acetylcholine in estimating the activities of the two cholinesterases separately (Mendel et al 1943), it was necessary to ascertain whether the inhibitions by Nu-1250 of the hydrolysis of these substrates and of acetylcholine are identical.

The results of these investigations appear below.

(1) The inhibition by Nu-1250 of the cholinesterases in the plasma and tissues of various species is summarized in Table 1, the values obtained with each concentration of the inhibitor representing the average of several determinations. It can be seen that Nu-1250 acts as a selective inhibitor of true cholinesterase. However, the margin between the sensitivities of the true cholinesterase and pseudo-cholinesterase in the plasma and tissues varies from species to species. In the case of human subjects (section A) the activity of true cholinesterase (erythrocytes) is at least a thousand times more sensitive to the inhibitory action of Nu-1250 than is the pseudo cholinesterase (plasma), with the tissues of the dog this margin drops to about twenty times, whilst in the case of the true cholinesterase and pseudo-cholinesterase of horse blood, a margin of approximately five times is obtained. The rat seemed ideal for *in vivo* experiments because the true cholinesterase in the plasma of this animal is at least a thousand times more sensitive to Nu-1250 than is the pseudo-cholinesterase.

Table 1 *Percentage inhibition by Nu-1250 of the cholinesterases in the plasma and tissues of various species**

Section	Enzyme source	Substrate		Concentration of Nu 1250 (M)								
		Nature	Concentration (M)	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	5 × 10 ⁻⁸	10 ⁻⁸	5 × 10 ⁻⁹	10 ⁻⁹
A	Human erythrocytes	Ach	0.0012	—	—	100	96	84	—	66	60	34
		Mch	0.03	—	—	—	—	86	—	—	56	—
	Human plasma	Ach	0.06	97	58	29	16	—	—	0	—	—
B	Horse erythrocytes	Ach	0.0012	—	—	—	100	97	93	58	—	—
	Horse plasma	Ach	0.06	—	—	100	97	70	66	—	—	—
C	Dog brain (nucleus caudatus)	Ach	0.0012	—	—	100	95	92	86	—	—	—
	Dog pancreas	Bch	0.006	—	—	88	83	53	14	—	—	—
	Dog plasma	Mch	0.03	—	—	—	—	93	87	—	—	—
		Bch	0.006	—	—	—	—	54	32	—	—	—
D	Fractionated rat plasma*											
	Precipitate (P)	Mch	0.03	—	—	100	100	100	91	82	69	13
		Ach	0.0012	—	—	—	—	—	—	—	72	—
	Supernatant fluid (S)	Bch	0.006	—	81†	65	45	31	24	6	—	—
		Ach	0.06	—	76†	—	43	—	—	—	—	—

* See Methods section for details of procedure and explanation of abbreviations used

† Nu 1250 concentration used here was $3 \times 10^{-5} M$

(2) Experiments with human erythrocytes, horse erythrocytes and the precipitate (P) from fractionated rat plasma (sections A, B and D of Table 1) showed that when acetyl- β -methylcholine was employed as substrate the inhibition of the activity towards that compound effected by Nu-1250 mirrored the inhibition displayed when the natural substrate acetylcholine was used. Hence, it was deemed valid to consider the inhibition of the activity towards acetyl- β -methylcholine as a reflexion of the inhibition of the acetylcholine hydrolysis brought about by true cholinesterase. Similar results with the supernatant fluid (S) from fractionated rat plasma (section D, Table 1), which had been shown to contain pseudo-cholinesterase only, justified substituting the inhibition of the activity towards benzoylcholine for the inhibition of the acetylcholine hydrolysis brought about by pseudo cholinesterase.

(B) *In vivo* experiments

Since the true cholinesterase in the plasma of rats was found to be about a thousand times more sensitive to the inhibitory action of Nu-1250 than the pseudo-cholinesterase (see Table 1, section D), the rat seemed most suitable for the *in vivo* experiments. The only difficulty appeared to be that a comparatively large volume of blood (2.5 ml) had to be obtained before the injection of the inhibitor in order to determine the normal levels of the cholinesterases in the plasma. However, Sawyer &

Everett (1946) have shown that in adult male rats both cholinesterases remain fairly constant over long periods of time, and preliminary experiments in the present series revealed that after the removal of 2.5 ml of blood from rats weighing about 300 g an interval of 2 weeks sufficed for the regeneration of both enzymes to previous levels. Therefore, adult male rats, weighing at least 300 g were used in the following experiments.

Blood (2.5 ml) was removed by heart puncture under light ether anaesthesia. The activities of the cholinesterases in the plasma obtained from this sample served as controls against which the magnitude of the inhibition of these enzymes was gauged, when an effective dose of Nu 1250 was injected 2 weeks later. The effectiveness of the dose was determined by the appearance of well defined symptoms indicative of acetylcholine accumulation, viz. chewing movements, yawning, and extensive and violent fibrillary twitching of the voluntary muscles. Upon their appearance the rats were killed by exsanguination through the carotid artery, and the blood was collected in a beaker containing sodium oxalate (c. 20 mg). The susceptibility of the complex between the cholinesterases and Nu-1250 to dissociation upon dilution necessitated the measurement of enzymic activity in the undiluted plasma of these animals.

Table 2 gives the cholinesterase levels in the plasma of six rats which displayed well defined symptoms of acetylcholine accumulation following the intraperitoneal injection of Nu-1250. It will be seen that the true cholinesterase is inhibited 75–89%, whereas the activity of the pseudo cholinesterase is but insignificantly affected. Thus,

Table 2 *Inhibition of the cholinesterases in the plasma of rats injected with Nu-1250*

(Activities are expressed as $\mu\text{l CO}_2$ evolved in 20 min by 1 ml plasma with Mch and Bch as substrates for true ChE and pseudo ChE, respectively)

Rat no	Wt (g)	Wt Nu 1250 injected (mg)	True cholinesterase			Pseudo cholinesterase		
			Activity before injection ($\mu\text{l CO}_2$)	Activity after injection ($\mu\text{l CO}_2$)	Inhibition (%)	Activity before injection ($\mu\text{l CO}_2$)	Activity after injection* ($\mu\text{l CO}_2$)	Inhibition (%)
1	330	0.066	52.5	13.2	75.0	49.2	48.0	2.5
2	335	0.066	44.0	6.0	86.0	36.0	32.0	11.0
3	350	0.070	45.0	7.2	84.0	29.0	27.3	6.0
4	300	0.080	44.3	4.8	89.0	34.6	30.4	12.0
5	360	0.158	43.0	5.2	87.5	35.0	31.2	10.0
6	395	0.170	44.5	6.0	86.5	44.3	37.3	15.0

* Corrected for CO_2 retention

in spite of its virtually undiminished activity, pseudo-cholinesterase is unable to prevent the effects of acetylcholine accumulation when the true cholinesterase is inhibited.

To exclude the possibility that the reaction of the experimental animals was due to an effect of Nu-1250 not related to acetylcholine accumulation, (+)-tubocurarine, which blocks the response of skeletal muscles to acetylcholine, was administered to a series of rats when the fibrillary twitching was at its height. The intraperitoneal injection of (+)-tubocurarine (1.25 mg/kg) arrested the fibrillation almost immediately, thus proving that the symptoms elicited by Nu-1250 were due to the accumulation of acetylcholine.

DISCUSSION

As Adams & Thompson (1948) have pointed out, cholinesterase inhibitors may be divided into three main groups: (1) compounds which inhibit pseudo- and true cholinesterase to the same extent, (2) compounds which act selectively on pseudo-cholinesterase, and (3) selective inhibitors of true cholinesterase. Group (1) includes eserine and prostigmine (Hawkins & Mendel, 1946), group (2) includes pyrazolone derivatives (Zeller, 1942), percarine (Zeller & Bissegger, 1943), tri-*o*-cresylphosphate (Mendel & Rudney, 1944), certain unauthenticated curare preparations (Harris & Harris, 1944), the prostigmine analogue Nu 683 (Hawkins & Gunter, 1946), diisopropyl fluorophosphonate (Hawkins & Mendel, 1947), and *N*-diethylaminoethylphenothiazine (2987 R P) (Gordon, 1948), group (3) includes caffeine (Zeller & Bissegger, 1943), certain 'nitrogen mustards', especially di-(2-chloroethyl)-methylamine (DDM) (Adams & Thompson, 1948) and the prostigmine analogue Nu-1250.

By selectively inhibiting pseudo cholinesterase *in*

in vivo Hawkins & Gunter (1946) demonstrated that this enzyme plays no essential role in the hydrolysis of acetylcholine in the animal body. Whether an auxiliary role might be assumed by pseudo-cholinesterase in cases where the activity of true cholinesterase is impaired could not be determined by these authors, because no suitable selective inhibitor of true cholinesterase was available at that time. The results outlined in the present paper, however, permit the conclusion that pseudo-cholinesterase is incapable of playing even an auxiliary role in preventing the accumulation of acetylcholine *in vivo*. Consequently, the appearance of symptoms indicative of acetylcholine poisoning following the administration of a cholinesterase inhibitor from any one of the aforementioned groups must be directly related to the action of the inhibitor on true cholinesterase. Moreover, an inhibition of approximately 75% of the activity of true cholinesterase must be exceeded before the onset of symptoms of acetylcholine accumulation can be expected, since the experiments of Gunter & Mendel (1945), Hawkins & Gunter (1946) and Hawkins & Mendel (1947) have shown that a surplus of true cholinesterase exists in the animal body. It would seem therefore that if attempts are made to correlate the pharmacological effects of a compound with its ability to act as a cholinesterase inhibitor, they will be of no avail, unless the cholinesterase inhibitory potency expresses the degree of inhibition of the true cholinesterase.

SUMMARY

1. The prostigmine analogue, *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Hoffmann-La Roche Nu-1250), was found to be a selective inhibitor of true cholinesterase.

2 Inhibition of true cholinesterase in rats brought about by the injection of Nu-1250 elicits symptoms indicative of acetylcholine accumulation, in spite of the undiminished activity of pseudo-cholinesterase. Thus, pseudo-cholinesterase is not essential to the hydrolysis of acetylcholine *in vivo*, as previous experiments have shown, nor is it capable

of assuming even an auxiliary role in this process when the activity of the true cholinesterase is impaired

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The Absorption of Vitamin A in Ruminants and Rats

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Drummond, Bell & Palmer (1935) and McCoord, Breese & Baum (1943) demonstrated an increased concentration of vitamin A in the lymph collected from the thoracic duct, after oral administration of the vitamin. Popper & Volk (1944) observed a fluorescence typical of vitamin A in the lacteals of the rat following dosage. Radice & Herraiz (1947) confirmed the results of Popper and claimed that they had observed a similar fluorescence in portal blood. These findings suggested that vitamin A may be absorbed by two different routes, as has been described for fats by Frazer (1946).

In the present study, both the portal blood and the lymph were examined as possible pathways of absorption, in order to ascertain the relative importance of the two routes. The experiments were performed on three species, oxen, sheep and rats, by dosing them with vitamin A and estimating the vitamin in systemic and portal blood, and in lymph

glands from various regions of the body. The samples were collected as soon as possible after slaughter of the animals.

EXPERIMENTAL

Treatment of animals

The animals were given vitamin A (5000 i.u./kg. body wt.) in the form of halibut liver oil, by mouth. Doses were prepared for bullocks and sheep by emulsifying the halibut liver oil with reconstituted separated milk by means of a Waring Blender. Rats, fasted for 12 hr., were dosed from a precision pipette with undiluted oil.

Bullocks. Fourteen Ayrshire and two Friesian bullocks weighing 150–250 kg. each were dosed at different times, ranging from 2 to 24 hr., before slaughter. The total bulk of the dose was 750 ml. Before dosing, samples of jugular blood were taken into oxalate, and after dosing, samples of jugular and portal blood were collected, usually within 2 and not exceeding 5 min. after slaughter. All blood

samples were oxalated to prevent clotting by mixing the blood at collection with a 10% oxalate solution to give a final dilution of 0.1%. Lymph glands from various intestinal regions and other parts of the body were removed, sliced and allowed to drain, vitamin A estimations were performed within 24 hr on the lymph thus obtained.

Sheep Nineteen adult sheep, weighing 60–90 kg each, were used, the dose of halibut liver oil was made up in a bulk of 400 ml and given 4–7 hr before slaughter. The procedure closely followed that for the bullocks, but no attempt was made to separate the mesenteric lymph glands according to their connexions with the various parts of the intestine.

Rats It was difficult to obtain from single rats enough material for accurate estimations, and therefore the experiments were performed by pooling the material from five rats. Albino and piebald rats weighing 200–250 g each were used and a group was killed at times varying from 1 to 8 hr after dosing. Systemic and portal blood (0.5 ml) was collected from each rat and the five samples pooled. It was impossible to collect the fluid from the lymph glands, and hence vitamin A estimations were performed on the whole of the mesenteric lymph tissue.

Chemical methods

Methods of extraction Plasma and lymph were treated according to the method of Yudkin (1941) which consists in precipitating the proteins with ethanol and extracting the vitamin with light petroleum. For estimations 2 ml of plasma or lymph were generally taken, although it was not always possible to obtain this amount from the lymph glands of the body. The method described by Glover, Goodwin & Morton (1947) for liver tissue was used for lymph tissues. They were ground with sand, dehydrated with anhydrous Na_2SO_4 and extracted with hot ether.

Estimations of carotene and vitamin A Colour intensities were measured in a single photocell absorptiometer, similar to that described by Evelyn (1939). Small Ogal cells (Tintometer Ltd.) of 1 cm depth and 17 ml capacity were used for the solutions. Carotene, when present, was estimated by measuring the yellow colour of the light petroleum extracts of the samples, using Wratten filter no. 47. Crystalline β carotene was used for calibration. The method of estimating vitamin A was similar to that described by Eden (1948), except for the following modifications. After adjusting the galvanometer to full scale deflection with chloroform as the blank, the cell containing 0.1 ml of the extract was reinserted into the absorptiometer. As the colour of the SbCl_3 reaction mixture fades rapidly, it was necessary to obtain a quick reading. Hence 0.4 ml of SbCl_3 reagent was blown into the cell so as to ensure rapid mixing and the reading was taken within 10 sec. A concentrate of vitamin A ester (Distillation Products, Inc.) containing 700,000 U.S.P. units/g was used for the standard reference curve. One U.S.P. unit was regarded as equivalent to one i.u.

Correction for carotenoids In the estimation of vitamin A in bullocks' plasma, which contains sufficient carotenoids to interfere with the Carr-Price SbCl_3 colour reaction, a correction was made by deducting one quarter of the carotene values expressed as i.u. from the original total vitamin A figures. Other materials examined contained only traces of carotenoids, and hence no correction was applied.

RESULTS

The results for bullocks, sheep and rats are presented in Tables 1, 2 and 3 respectively.

Blood After dosing, the vitamin A concentration of both the systemic and portal blood rose in all three species approximately 80% above the levels before dosing. In general, the vitamin A values of systemic blood were higher than those of portal blood. The actual figures for portal and systemic blood after dosing were 147 and 162 for bullocks, 187 and 220 for sheep and 224 and 234 i.u./100 ml for rats, respectively.

Lymph The vitamin A content of the lymph draining the small intestine of the dosed animals was on the average about ten times that of the undosed animals. There was a rise from 225 to 1500 i.u./100 ml in bullocks, from 100 to 4830 i.u./100 ml in sheep and from 0.4 to 3.2 i.u./rat. On the other hand, the differences between the body lymph of dosed and undosed animals were within the experimental error of the method, which was greatly increased by the small amounts available for estimations.

An attempt was made to find out which part of the intestine was mainly responsible for the absorption of the vitamin A, by analyzing lymph from various parts of the gut. It was found that with one exception lymph obtained from the glands draining the duodenum had a higher vitamin A content than the lymph from jejunum or ileum (Table 1). On the other hand, the lymph draining the large intestine showed no rise above that of the body lymph (169 and 175 i.u. of vitamin A/100 ml respectively) indicating that no marked absorption of the vitamin had occurred from this region of the gut; similarly, no absorption could be observed from the stomach.

DISCUSSION

Our experiments have shown that in ruminants and rats vitamin A is absorbed through the intestinal lymph. This agrees with results obtained on other animals by Drummond *et al.* (1935), McCoord *et al.* (1943), Popper & Volk (1944) and Radice & Herraiz (1947).

From our experiments it seems that most of the absorption occurred from the upper part of the intestine (Table 1). Popper & Volk (1944) also contend that the upper two thirds of the intestine is the most effective region of absorption.

We have been unable to confirm the work of Radice & Herraiz (1947) on portal absorption. After dosing, the average figures in our experiments for portal blood showed no marked difference from those for systemic samples. It is possible that the period of absorption through the portal blood may

Table 1 *Concentration of vitamin A in plasma and lymph of bullocks after administration of 5000 i u /kg of body weight*

Vitamin A (1 u /100 ml)								
Animal no	Time killed (hr) after dose	Plasma						
		At slaughter			Lymph (at slaughter)			
		Before dosing	Systemic		Non- intestinal	Intestinal		
				Portal		Duodenum	Jejunum	Ileum
Dosed animals								
1	2	64	146	143	67	1130	305	—
2	5	84	212	225	245	1250	2870	1430
3	8	91	204	209	115	2900	1600	405
4	12	81	145	140	237	2030	1330	300
5	12	59	100	101	—	581	305	150
6	16	69	139	79	144	1480	658	215
7	16	85	166	100	95	1270	1020	170
8	24	66	145	115	—	2550	1660	—
9	24	57	170	176	132	202	192	—
10	24	61	189	183	185	1680	560	363
	Average	72	162	147	175	1500	1030	460
Undosed animals								
11	0	65	65	72	146	178	178	146
12	0	108	100	109	114	320	190	115
	Average	87	83	90	130	225	185	130

Table 2 *Concentration of vitamin A in plasma and lymph of sheep after administration of 5000 i u /kg of body weight*

		Vitamin A (i u /100 ml)				
Animal no	Time killed (hr) after dose	Plasma			Lymph (at slaughter)	
		Before dosing	At slaughter		Non- intestinal	Intestinal
			Systemic	Portal		
Dosed animals						
1	4	138	344	170	104	1,020
2	4	112	410	355	75	9,640
3	4	103	328	156	21	4,140
4	4	138	159	188	15	8,880
5	4	172	182	175	—	14,000
6	4	170	143	120	—	2,800
7	5	108	149	140	39	610
8	5	128	267	172	9	1,300
9	5	82	197	118	95	3,500
10	5	107	182	160	57	7,500
11	5	107	215	170		
12	7	86	126	350	49	1,020
13	7	122	182	143		
Average		121	220	187	52	4,830
Undosed animals						
14	0	170	143	120	—	314
15	0	86	71	52	22	47
16	0	86	74	47		
17	0	77	84	54		
18	0	107	116	48	55	71
19	0	107	77	57		
Average		105	94	63	35	100

Table 3 Concentration of vitamin A in plasma and lymph of rats after administration of 5000 i u /kg of body weight

Group no	Time killed (hr) after dose	Vitamin A		
		1.u /100 ml plasma		1 u /rat lymph tissue (intestinal)
		Systemic	Portal	
Dosed animals				
1	2	156	250	—
2	2	240	255	3 0
3	2	215	162	3 0
4	2	638	470	3 7
5	3	243	220	2 3
6	5	145	147	—
7	5	133	114	3 7
8	5	225	245	2 5
9	8	113	153	3 3
Average		234	224	3 2
Undosed animals				
10	0	104	96	—
11	0	104	117	0 4
12	0	104	104	0 4
Average		104	105	0 4

be short, and that in these experiments any rise was missed. This is, however, considered unlikely as various periods between 1 and 24 hr were allowed for absorption. Another possibility is that the absorption rate of vitamin A through the portal blood is low, causing a rise too small to be detected by the present methods of estimation. As the portal circulation during digestion is rapid, the total amounts of vitamin A thus absorbed may be quite considerable. The difference between the results obtained by Radice & Herraiz (1947) and those in this experiment may also be influenced by the fact that they gave a dose forty times larger.

A possible criticism of the present investigation is that the amounts of vitamin A administered were considerably larger than those normally taken in

food. However, in experiments not yet published, we obtained similar results when newborn calves were fed artificial colostrum, fortified by amounts of vitamin A within the range occurring in natural colostrum.

From our results we have been unable to obtain any conclusive evidence that vitamin A is carried from the intestine by the portal blood. On the other hand, the lymph draining the small intestine seems to be the main pathway by which the vitamin A reaches the general circulation.

SUMMARY

1. Bullocks, sheep and rats were dosed with 5000 i u of vitamin A/kg body weight and slaughtered 1–24 hr after dosing. On slaughter, vitamin A was estimated in systemic and portal blood, and also in the lymph or lymphatic tissues obtained from glands of the intestinal and other regions of the body.

2. In all three species, the lymph or lymph glands of the intestine contained considerably more vitamin A in dosed than in undosed animals. The average values for sheep and bullocks after dosing were 4830 and 1500 i u of vitamin A/100 ml and 180 and 225 i u /100 ml for animals not dosed. This marked rise was not found in the body lymph after dosing, the concentrations being only 52 and 175 i u /100 ml respectively. The major part of this absorption seems to take place in the upper part of the intestine.

3. The vitamin A content of portal blood and of systemic blood rose after dosing, but the average figures for the portal blood were if anything slightly lower than those for systemic.

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The Decarboxylation of *o*-Hydroxyphenylalanine

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Two new substrates of the mammalian enzyme DOPA decarboxylase have recently been described, *L-m*-hydroxyphenylalanine and *L-2,5*-dihydroxyphenylalanine (Blaschko & Sloane Stanley, 1948). Both these amino acids have, like *L-3,4*-dihydroxyphenylalanine, the phenolic hydroxyl group in the meta position relative to the side chain, and it was, therefore, concluded that it is the presence of this group in these three substances which enables them to be substrates.

Tyrosine is not a substrate of the enzyme, but it is not known if the presence of a phenolic hydroxyl group in the ortho position is of importance for substrate specificity. In the experiments described in this paper the decarboxylation of *o*-hydroxyphenylalanine by mammalian tissue extracts has, therefore, been studied. At the same time, a few observations on the action of amino acid oxidases on *o*-hydroxyphenylalanine are reported.

MATERIAL AND METHODS

The observations were made on a sample of *DL-o*-hydroxyphenylalanine for which I am grateful to Dr A. Neuberger.

For the experiments with decarboxylase, extracts were freshly prepared by grinding the organs with sand and adding 1 ml of 0.067 M-sodium phosphate buffer (pH 7.4)/g of tissue. The supernatant fluid after 5 min centrifuging was used. It was assumed that 1 ml of extract contained the activity of 0.5 g of tissue. The rate of CO₂ formation was measured manometrically as previously described (Blaschko, 1942), all the data given are corrected for CO₂ retention.

For the study of amino acid oxidases two preparations were used. *a* Amino acid oxidase was obtained from an acetone dried powder of pig kidney. The powder (400 mg) was left to stand at room temperature for 1 hr with 4 ml of 0.067 M sodium phosphate buffer (pH 7.4), of the supernatant fluid after centrifuging 0.6 ml was used/flask. The second preparation was a sample of cobra venom which contained a powerful *L*-amino acid oxidase described by Zeller & Maritz (1944).

RESULTS

(a) Decarboxylation of *o*-hydroxyphenylalanine

Extracts from guinea pig kidney and rat liver were used. These organs contain DOPA decarboxylase. A solution of *DL-o*-hydroxyphenylalanine, when incubated with these extracts under anaerobic

conditions at 37.5°, gave rise to CO₂, the rate of formation of which was measured. The total amount of CO₂ formed suggested that only one stereoisomer was decarboxylated. For instance, in one experiment with 0.8 g of guinea pig kidney the reaction had come to a standstill after 45.5 ml of CO₂ had been formed from 0.4 ml of 0.01 M *DL-o*-hydroxyphenylalanine added. This corresponds to a formation of 0.51 mol CO₂/1.0 mol *DL* amino acid. In view of what is known of the stereo specificity of DOPA decarboxylase (Holtz, Heise & Ludtke, 1938; Blaschko, 1942; Blaschko, Holton & Sloane Stanley, 1949) it seems safe to assume that it is the *L*-isomer of *o*-hydroxyphenylalanine which is decarboxylated.

The rate of decarboxylation of *o*-hydroxyphenylalanine is a little slower than that of 3,4-dihydroxyphenylalanine. The rates of formation of CO₂ from *o*-hydroxyphenylalanine, 3,4-dihydroxyphenylalanine and *m*-hydroxyphenylalanine were compared, in one experiment guinea pig kidney extract was used, in the second rat liver extract. Each flask contained 0.4 ml of 0.02 M-*DL* amino acid, the total volume was 2 ml. During the first experiment in which each flask contained extract from 0.15 g of guinea pig kidney, the amounts of CO₂ formed in the first 6 min were

	μl
With 3,4-dihydroxyphenylalanine	43
With <i>o</i> -hydroxyphenylalanine	32
With <i>m</i> -hydroxyphenylalanine	35

In the second experiment, with extract from 0.8 g of rat liver, the corresponding figures for CO₂ formation were

	μl
With 3,4-dihydroxyphenylalanine	52
With <i>o</i> -hydroxyphenylalanine	40
With <i>m</i> -hydroxyphenylalanine	50

In different experiments the relative rates of decarboxylation have differed slightly, but the rate with 3,4-dihydroxyphenylalanine as substrate was always slightly greater than with either *o*- or *m*-hydroxyphenylalanine.

DOPA decarboxylase is the catalyst responsible for the decarboxylation of *m*-hydroxyphenylalanine (Blaschko *et al.* 1949), the experiments described above suggested that *o*-hydroxyphenylalanine was another substrate of the same enzyme.

This was confirmed in a substrate competition experiment the initial rate of CO₂ formation in the presence of both *o*- and *m*-hydroxyphenylalanine was about the same as with either substrate alone. With the extract from 0.15 g of guinea pig kidney the amounts of CO₂ formed in the first 9 min were

	μ l
With 0.008 M <i>m</i> -hydroxyphenylalanine	28.5
With 0.008 M <i>o</i> -hydroxyphenylalanine	35
With both amino acids	31

(b) *Enzymic oxidation of o-hydroxyphenylalanine*

An extract of pig kidney powder consumed O₂ in the presence of DL-*o*-hydroxyphenylalanine. This is taken as an indication that DL-*o*-hydroxyphenylalanine is a substrate of D-amino acid oxidase. The rate of oxidation of *o*-hydroxyphenylalanine was compared with those of *m*-hydroxyphenylalanine and methionine, the initial concentrations of the amino-acids were 0.03 M. The amounts of O₂ consumed in the first 12 min were

	μ l
With DL- <i>o</i> -hydroxyphenylalanine	8
With DL- <i>m</i> -hydroxyphenylalanine	16
With DL-methionine	198

o-Hydroxyphenylalanine is also a substrate of the L-amino-acid oxidase of cobra venom. The rates of O₂ uptake with the same three amino-acids have been compared in an experiment in which each flask contained 1.2 mg of cobra venom, the initial amino-acid concentrations were 0.028 M. The O₂ uptakes during the first 15 min of the reaction were

	μ l
With DL- <i>o</i> -hydroxyphenylalanine	10
With DL- <i>m</i> -hydroxyphenylalanine	54
With DL-methionine	90

DISCUSSION

Metabolism of o-hydroxyphenylalanine

DL-*o*-Hydroxyphenylalanine was first synthesized by Blum (1908), who showed that, like *m*-hydroxyphenylalanine, it did not give rise to homogentisic acid in human alcaptonuria. He found that in a normal subject about one third of a dose of 5 g was excreted as *o*-hydroxyphenylacetic acid in the urine. *o*-Hydroxyphenylacetic acid was also found in the urine of rabbits after feeding either *o*-hydroxyphenylalanine or *o*-hydroxyphenylpyruvic acid (Flatow, 1910). Subsequently the experiments of Blum (1908) and Flatow (1910) were often quoted as proof for Neubauer's scheme of degradation of amino acids by oxidative deamination. The results described in the experimental part of this paper can be considered as giving support to this scheme. The

two stereoisomers of *o*-hydroxyphenylalanine were found to be substrates of amino-acid oxidases, and it therefore seems likely that *o*-hydroxyphenylacetic acid can arise from the DL-amino-acid via *o*-hydroxyphenylpyruvic acid.

The present findings make it necessary, however, to consider another possible pathway of degradation of *o*-hydroxyphenylalanine, which would also lead to *o*-hydroxyphenylacetic acid. This pathway leads from L-*o*-hydroxyphenylalanine via *o*-hydroxyphenylethylamine and *o*-hydroxyphenylacetaldehyde to *o*-hydroxyphenylacetic acid. The first of these steps is the decarboxylation reaction described in the first part of this paper, the second step is the oxidation of *o*-hydroxyphenylethylamine by amine oxidase, a reaction described by Randall (1946). The oxidation of the aldehyde formed in the oxidative deamination of *o*-hydroxyphenylethylamine to the corresponding acid—*o*-hydroxyphenylacetic acid—is parallel to the breakdown of closely related substances. The fate of *o*-hydroxyphenylethylamine considered in this scheme is in fact similar to that of tyramine. Ewins & Laidlaw (1910) have shown that tyramine is excreted as *p*-hydroxyphenylacetic acid in the dog, and that the same end product is found when the livers of cats and rabbits are perfused with tyramine. These observations found their explanation in the discovery of amine oxidase (Hare, 1928; Bernheim, 1931).

It is not known to what extent these two pathways contribute to the formation of *o*-hydroxyphenylacetic acid from *o*-hydroxyphenylalanine. The same is true for the breakdown of *m*-hydroxyphenylalanine. *m*-hydroxyphenylacetic acid is found in the urine when *m*-hydroxyphenylalanine is fed (Blum, 1908; Flatow, 1910), *m*-hydroxyphenylalanine is also a substrate of amino acid oxidases as well as of DOPA decarboxylase (Blaschko *et al.* 1949).

Substrate specificity of DOPA decarboxylase

The substrate specificity of DOPA decarboxylase can now be more precisely defined. It has been shown that the presence of two phenolic hydroxyl groups is not required, the presence of one such group is essential. However, the position of this group in relation to the side chain is of importance, a hydroxyl group in the para position to the side chain is not sufficient, but the presence of a hydroxyl group in either the meta or in the ortho position is sufficient.

A similar study of substrate specificity has been carried out in this laboratory for the L-tyrosine decarboxylase of *Streptococcus faecalis* R (Sloane Stanley, 1949). This enzyme has a completely different pattern of affinities, it has a marked

preference for tyrosine, it acts less readily on *m*-hydroxyphenylalanine and has practically no affinity for *o*-hydroxyphenylalanine

The bacterial and the mammalian enzyme are closely related, both are pyridoxalphosphate-proteins (Gunsalus, Bellamy & Umbreit, 1944, Gale, 1946, Green, Leloir & Nocito, 1945, Blaschko, Carter, O'Brien & Sloane Stanley, 1948) Their different affinities for tyrosine and its two isomers must, therefore, be due to structural differences of the enzyme proteins The observations reported here show that the position of the phenolic hydroxyl group is one of the factors which govern the enzyme-substrate relationships of the two decarboxylases

SUMMARY

1 Extracts of rat liver and guinea pig kidney form carbon dioxide when incubated anaerobically with *o*-hydroxyphenylalanine, it is suggested that *L o*-hydroxyphenylalanine is a substrate of DOPA decarboxylase

2 *o*-Hydroxyphenylalanine is a substrate of the *D*-amino-acid oxidase of pig kidney

3 *o*-Hydroxyphenylalanine is a substrate of the *L* amino acid oxidase of cobra venom

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Reducing-group Production from Starch by the Action of α - and β -Amylases of Barley Malt. Activity of α - and β -Amylases

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Recent proposals for the determination of α amylase of barley malt (Graesser & Dax, 1946, Preece, 1947, 1948) involve differential heat inactivation at 70° in the presence of calcium ions, followed by standardized conversion of a soluble starch substrate (American Society of Brewing Chemists, 1944, Institute of Brewing, 1933), with determination of reducing groups by ferricyanide titration or by Fehling's titration respectively Thus, β -amylase is heat-inactivated and the observed saccharification of the starch is brought about by α -amylase alone (cf Kneen, Sandstedt & Hollenbeck, 1943) Total saccharification by the joint action of the α - and β amylases present in the malt being readily determined as a measure of diastatic power by a similar method with the heat inactivation omitted, saccharification by β -amylase should be obtainable by difference

These proposals involve a number of assumptions Thus, it is necessary to be able to work under such conditions for starch conversion that the production of reducing groups shows a linear relationship with enzyme concentration and with duration of action, and also to know that the reactions of the two components are, in fact, truly additive in the reaction range used The published data relating to these points have been developed under a wide variety of experimental conditions Thus, of the more recent work, Kneen & Sandstedt (1941), using 2% (w/v) soluble starch solution for hydrolysis at 30°, find linearity between enzyme concentration and reducing-group production (ferricyanide method) up to 40% hydrolysis for the joint action of the two enzymes, and up to 30% for β amylase alone, for α -amylase they found no region of linearity Further, it was shown that, under the experimental condi-

tions used, saccharification by the two components was additive for a wide range of ratios of β - to α -amylase saccharogenic activity, deviating slightly, however, when this ratio was less than 2 Claus (1947), on the other hand, investigating β -amylase activity at pH 5.5 and various temperatures, with 20% maize starch paste as substrate, found reducing-group production (ferricyanide ceric sulphate titration) to be a zero-order reaction up to 18% conversion Freeman & Hopkins (1936), at 25°, pH 4.6, and with 2% (w/v) soluble starch solution, found that with low concentrations of the two enzymes saccharification (iodometric titration) was additive, though with higher concentrations of β -amylase the observed saccharification might exceed that calculated from the two component rates

Although there is thus some agreement that an additive reaction is, in general, concerned—at least when the total hydrolysis achieved is not great—the data quoted do not afford a precise guide to conditions operating in the widely used standard methods for diastatic power determination of the Institute of Brewing (1933) and of the American Society of Brewing Chemists (1944) and in the proposed modification for determination of the individual enzymes Accordingly, the problem has been reinvestigated using 2% and 1% (w/v) soluble starch substrates at 21° and pH 4.6, as a necessary preliminary to a more extended study of the joint action of the two enzymes

EXPERIMENTAL

Reducing group determination In a modification of the Hagedorn Jensen method, ferricyanide carbonate reagent was used under conditions similar to those described by Lampitt, Fuller, Goldenberg & Green (1947), but with the range increased to determine a maximum of 18 mg of reducing groups, calculated as anhydrous maltose Thus, the ferricyanide reagent contained 33.0 g $K_3Fe(CN)_6$ /500 ml, and the carbonate 42.4 g anhydrous Na_2CO_3 /500 ml For titration, 0.025N $Na_2S_2O_3$ was used, and the other reagents and the general conditions of working were as described by Lampitt *et al* (1947) Standardized against a sample of maltose of known purity, under conditions of salt concentration identical with those encountered in the starch conversions, a linear relationship holds between maltose concentration and thiosulphate titration figures, 1 ml 0.01N thiosulphate is equivalent to 0.368 mg of maltose, a figure differing materially from that of Lampitt *et al* (1947), but agreeing with that of the American Society of Brewing Chemists' method (1944), in which the relative proportions of ferricyanide, carbonate, and reducing groups during the reaction are substantially the same as those now used

β Amylase preparation In a modification of the method of Hopkins, Murray & Lockwood (1946), ground barley was extracted with 2.5 times its weight of 20% (v/v) ethanol for 30 min with stirring, the mixture filtered and the concentration of ethanol adjusted to 50% (v/v) After allowing to stand for 18 hr the precipitate was

filtered off and discarded, and the concentration of ethanol in the filtrate adjusted to 80% (v/v) The precipitate, taken to dryness in the usual way and tested by the method of Preece (1947), proved to contain not more α amylase than would produce 1/750 of the saccharification due to β amylase (Table 1)

Table 1 *Effect of maintaining solutions of α - and β -amylases at 70° in presence of Ca^{++}*

(Samples of the enzyme solutions were allowed to act on 1% (w/v) soluble starch solution at 21° and pH 4.6 for 30 min)

Time of maintenance at 70° (min)	Reducing group production (calculated as mg maltose)	
	α Amylase	β Amylase
0	295.8	296.7
15	280.7	0.4
30	258.1	0.4
45	237.6	0.4

α Amylase preparation For this preparation a barley malt was used which had been specially dried at a temperature not exceeding 45° The ground malt was extracted for 3 hr with 20% (v/v) ethanol, and the filtrate adjusted to an ethanol concentration of 60% (v/v) The separated precipitate was taken to dryness to remove ethanol, redissolved in water and, after adding 0.2 g calcium acetate/100 ml, maintained at 70° for 15 min After cooling and removal of coagulated material, the α -amylase was reprecipitated and taken to dryness β -Amylase activity could not be detected in such preparations (Table 1)

Soluble starch substrate The sample of soluble starch used contained 0.5% of reducing groups (as maltose), solutions were prepared containing (a) 1.111 g (dry substance)/100 ml, or (b) 2.222 g/100 ml, acetate buffer being added in each case to give a pH value of 4.6 Solutions were adjusted before use to 21°

Starch conversions Conversions of both 1 and 2% (w/v) starch solutions were carried out, 90 ml of the bulk solution being used in each case The unit of enzyme activity chosen was that amount of enzyme preparation which would produce in 15 min reducing groups equivalent to 12.5 mg of maltose from 1 g of soluble starch in a total vol of 100 ml at pH 4.6 and 21° The weights of the initial α and β amylase preparations containing 1 unit of activity were 0.8 and 0.2 mg respectively Later preparations were somewhat more active The unit concentrations are symbolized as 1 α and 1 β respectively, and the approximate relative concentrations used are shown in Tables 2 and 3 Samples of solutions containing the appropriate amounts of enzyme, previously adjusted to 21°, were added to 90 ml portions of the starch solution, and the total volume in each case adjusted to 100 ml At appropriate intervals, samples of the conversions were pipetted into sufficient aqueous NaOH to give a total volume of 10 ml at pH 10.5–11, reducing group determinations were then carried out

RESULTS

Results in Tables 2 and 3, which are corrected for reduction due to the starch itself and to the enzyme solution, are expressed in terms of theoretical

Table 2 *Liberation of reducing groups in 1% (w/v) soluble starch solution by varying proportions of α - and/or β amylase*

(Temp, 21°, pH, 4.6, results expressed as percentage of theoretical maltose)

Approximate relative concentration of enzymes	Reaction time (min)					
	15	30	45	60	120	180
1 α	1.2	2.4	3.3	4.3	8.1	11.4
2 α	2.2	4.2	6.1	7.8	13.8	19.5
3 α	3.7	6.8	10.2	13.6	21.1	29.0
4 α	4.9	9.5	13.8	18.0	30.1	34.2
5 α	6.0	11.7	16.5	19.1	31.2	36.2
1 β	1.3	2.5	3.6	4.7	8.8	12.4
2 β	2.6	5.2	7.8	10.4	18.8	25.3
3 β	4.2	8.1	11.9	15.5	26.4	31.4
4 β	5.6	10.8	15.6	20.3	32.7	39.6
5 β	7.0	13.8	19.3	24.2	36.2	41.6
1 α + 4 β Obs	6.8	12.1	19.1	24.7	43.7	55.1
Calc	6.8	13.2	18.9	24.6	40.8	51.0
2 α + 3 β Obs	6.3	12.4	18.0	22.4	39.6	51.2
Calc	6.4	12.3	18.0	23.3	40.2	50.9
3 α + 2 β Obs	6.3	12.8	18.4	24.0	40.0	50.0
Calc	6.3	12.0	18.0	24.0	39.9	54.3
4 α + 1 β Obs	6.2	13.1	18.0	22.8	37.6	45.4
Calc	6.2	12.0	17.4	22.7	38.9	46.6

Obs = observed, Calc = calculated

Table 3 *Liberation of reducing groups in 2% (w/v) soluble starch solution by varying proportions of α - and/or β amylase*

(Temp, 21°, pH, 4.6, results expressed as percentage of theoretical maltose)

Approximate relative concentration of enzymes	Reaction time (min)					
	15	30	45	60	120	180
1 α	0.7	1.3	1.9	2.4	4.5	6.2
2 α	1.3	2.7	4.0	5.4	9.8	14.3
3 α	2.1	4.0	5.8	7.3	12.8	19.0
4 α	2.6	4.7	6.9	9.5	18.1	24.7
5 α	3.2	6.5	9.1	11.7	22.4	29.2
1 β	0.8	1.5	2.2	2.9	5.8	8.5
2 β	1.6	3.2	4.6	6.2	11.6	16.5
3 β	2.4	4.9	7.4	9.5	17.3	24.2
4 β	3.0	5.9	8.7	11.4	20.1	26.8
5 β	4.2	8.1	11.7	15.2	27.6	36.2
1 α + 4 β Obs	3.5	7.2	10.4	13.0	24.4	31.8
Calc	3.7	7.2	10.6	13.8	24.6	33.0
2 α + 3 β Obs	3.7	7.7	10.8	13.5	26.6	34.4
Calc	3.7	7.6	11.4	14.9	27.1	38.5
3 α + 2 β Obs	3.8	7.4	10.4	12.7	25.1	31.5
Calc	3.7	7.2	10.4	13.5	24.4	35.5
4 α + 1 β Obs	3.2	6.5	9.7	12.6	22.7	30.0
Calc	3.4	6.2	9.1	12.4	23.9	33.2

Obs = observed, Calc = calculated

maltose, i.e. reducing groups calculated as maltose are returned as a percentage of the maltose theoretically obtainable by complete hydrolysis of the starch, it being assumed that 1 g of starch would yield 1.055 g of maltose. Direct comparison is possible of pairs of results (observed and calculated) bracketed together at the foot of each table, as the three determinations involved were performed simultaneously and using the same preparations of enzyme solutions. Direct comparison between

results with other proportionate enzyme concentrations is less exact, as this involves comparison of enzyme solutions necessarily prepared on different occasions.

DISCUSSION

Examination of the results given in Tables 2 and 3 indicates that, under the conditions used, a linear relationship between reducing-group production and time of reaction persists for β -amylase up to the

production of approximately 15 % of theoretical maltose. This accords closely with the observation of Claus (1947), but is substantially lower than the limit of 30 % given by Kneen & Sandstedt (1941) for linearity between reducing-group production and enzyme concentration, for the latter relationship, however, the present results give linearity up to approximately 25 % suggesting, during the hydrolysis, a falling off in enzyme activity proportional to amylose concentration. Hence, comparison of two β amylase solutions giving conversions in the range 15–25 %, while allowing correct relative results, will tend to give absolute results which are somewhat lower than the true values.

With α -amylase the relationships are less simple. Thus it may be agreed that true linearity with time does not occur. However, so long as the reaction time does not exceed 30–45 min, the departure from linearity up to 10 % conversion is so small that very useful comparative measurements can be made in this range, in practice, little error is incurred if linearity is assumed up to 15 %. With α -, as with β -amylase, proportionality between enzyme concentration and reducing-group production holds with considerable accuracy up to 25 % hydrolysis. Thus, for comparison of the activities of two α amylase solutions, conversions up to 25 % may be employed, though for individual measurements for routine purposes a limit of 10–15 % should not be exceeded.

For joint action, linearity up to 40 % hydrolysis is usually assumed (Kjeldahl's law), but this assumption is not justified with the present methods. For the present results, proportionality between reducing group production and time ceases in the range 20–25 % hydrolysis for the whole series of β α ratios employed and for both 1 % (w/v) and 2 % (w/v) soluble starch substrate. On the other hand, the additive relation in joint action can only thus be stated in simple terms: reducing group production by α and β -amylases acting together is truly additive during the linear phase of joint action. When linearity is departed from, an additive relation may persist, depending *inter alia* on substrate concentration and the β α ratio. Thus, with 1 % (w/v) soluble starch, a β α ratio of 4 : 1 gives additive hydrolysis up to 25 % theoretical maltose production, thereafter in the range studied the observed hydrolysis exceeds that calculated as the sum of individual actions. With α - and β -amylase concentrations of similar reducing group-producing potentialities, the joint action is still additive at 40–50 % hydrolysis. With a β α ratio of 1 : 4, there is a tendency for the observed hydrolysis beyond 25 % to be somewhat below that calculated, a tendency which is seen with all the investigated ratios using 2 % (w/v) soluble starch substrate.

Therefore, if the described ferricyanide technique is used for amylase determination, hydrolyses in

a period preferably not greater than 30 min and not exceeding 15, 10–15 and 20–25 %, production of theoretical maltose should be employed for β -, α -, and joint action respectively. Applied to brewery malt extracts, in which the β α ratio is usually approx 4 : 1 (Preece, 1947), determination of α amylase activity and joint action (diastatic power) within the prescribed limits allows β -amylase activity to be obtained by difference. It would appear that, under the conditions described, α - and β -amylases function quite independently of one another.

It has recently been claimed by Blom & Røsted (1947) that the use of the ferricyanide method for determining reducing groups in solutions containing starch-conversion products tends to give high results as compared with copper-reduction methods. Thus, a solution which gave 42.0 % theoretical maltose by ferricyanide reduction gave 34.9 % with copper tartrate reagent (Fehling's solution), the surprisingly large discrepancy is probably due to a partial oxidative rupture of glucosidic bonds in oligosaccharides and polysaccharides. In view of the increasingly widespread use of modifications of the Hagedorn-Jensen method, because of its simplicity of operation and the high reproducibility of results, it is clearly important to know whether the suggested oxidative disruption of glucosidic linkages militates against obtaining accurate measurements. However, the method has been used with success by the American Society of Brewing Chemists (1944), results of diastatic power determination being returned as 'maltose equivalent' or, after dividing this figure by 4, as 'degrees Lintner', the same divisor is employed when Fehling's solution is used for reducing-group determination, and it must be remembered that the definition of 'degrees Lintner' depends fundamentally on the use of Fehling's solution. Whether a figure somewhat greater than 4 should be taken as divisor following the use of ferricyanide is at present under investigation, but apart from this possible objection there is little doubt of the superiority of the ferricyanide method for this type of work, the end point is sharper than with Fehling's solution (especially for α -amylase conversions), and no difficulties arise in carrying out enzyme and substrate blanks.

SUMMARY

1. Using 1 % (w/v) or 2 % (w/v) soluble starch as substrate at pH 4.6 and 21°, reducing-group production (assessed by ferricyanide titration) by β -amylase shows a linear relationship with time up to 15 % hydrolysis, and by the joint action of β - and α amylase a similar relationship up to 20–25 % hydrolysis. With α amylase alone true linearity is lacking, but departure from linearity up to 10–15 % hydrolysis is slight.

2 With β α ratios varying from 4:1 to 1:4, the action of the two enzymes is truly additive up to 20–25% hydrolysis, beyond this point it may be equal to, greater than or less than the sum of

individual actions, depending on substrate conditions

3 The significance of these facts in the assessment of amylolytic activity is discussed

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Changes in the Extracellular and Intracellular Fluid Phases of Muscle During Starvation and Dehydration in Adult Rats

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It has been shown in a previous paper (Dicker, 1948) that rats fed on a protein deficient diet develop tissue oedema very rapidly, i.e. the extracellular fluid phase of muscle and liver, as estimated in terms of chloride space, increases. The oedema starts at a time when the plasma protein concentration and the plasma colloid osmotic pressure are still normal. It seemed, therefore, of interest to investigate changes in the extracellular fluid phase of muscle in rats undergoing inanition or dehydration over short periods in which the plasma protein concentration was not decreased.

METHODS

Experimental animals Adult male and female albino rats were used, of body wt. 250–320 g.

Diet Some weeks before the experiment the animals were fed on a commercially prepared diet containing wheat offal 17.7, ground barley 8.8, white fish meal 4.5, meat and bone meal 8.8, dried skimmed milk 14.0, dried yeast 1.2, salt 0.4, and cod liver oil 0.4%. The total N content of the diet amounted to 3.26%, and its water content to 10.5%. The mineral content by analysis was 0.344 g Cl, 0.210 g Na, 4.100 g K/100 g and the calorific value 306.0 cal/100 g. During the period of experimentation the same type of food, but completely dehydrated, was given to one series of rats.

Analytical procedures The following data were determined in each rat: (a) the content of water, chloride, sodium, potassium and nitrogen of heparinized plasma (in some cases, the urea concentration of plasma was also

estimated), (b) the content of water, chloride, sodium and potassium of muscle samples (the muscle used was the rectus abdominis), (c) the concentration of Cl^- , Na, K and N in the urine, urea and NH_3 in the urine were estimated in some cases.

Water content, Cl^- , Na and K concentrations were estimated in tissue and plasma samples, in the same manner and by the same methods as described in a previous communication (Dicker, 1948). Urea in plasma was estimated colorimetrically according to the method of Lee & Widdowson (1937), and the N content of plasma was determined by a micro Kjeldahl method.

In the urine, Cl^- was estimated according to Volhard (1878), and Na and K by the method of McCance & Shipp (1933). NH_3 was adsorbed on permutit and estimated after nesslerization (Fohn & Bell, 1917), and the urea determination followed the method of Scott (1940). The specific gravity of urine was determined in the apparatus of Heller (1940), using a mixture of carbon tetrachloride and light petroleum. All data for muscle are expressed per 100 g fat-free tissue (see Hastings & Eichelberger, 1937).

Estimation of the extracellular and intracellular fluid phases of muscle The extracellular and intracellular fluid phases of muscle were calculated on the assumption that all the Cl^- is extracellular, and that its concentration is that of an ultrafiltrate of serum (Fulton, 1947). The volume of the extracellular fluid phase was determined by calculation (Hastings & Eichelberger, 1937).

The amount of potassium in the intracellular fluid phase was calculated as follows:

$$m \text{ equiv intracellular K/kg muscle} = (\text{K})_M - (\text{H}_2\text{O})_F \times (\text{K})_F,$$

where $(\text{K})_M = m \text{ equiv K/kg muscle}$, $(\text{K})_F = m \text{ equiv}$

K/kg extracellular water $= 0.95 \times (K)_s$, where $(K)_s = m$ -equiv K/kg plasma water, $(H_2O)_F = g$ extracellular water/ kg muscle $= 0.99 \times F$, where $F =$ extracellular fluid phase as calculated from the chloride space. The amount of Na in the intracellular fluid phase was similarly calculated (Fulton, 1947).

Response to water administration (water diuresis) Starved and dehydrated rats were given an amount of water equal to 5% of their body wt by stomach tube, and their urinary excretion was compared with that of normal rats. The evening before the tests the normal rats were deprived of food and water. Urine was collected in graduated cylinders, the urinary volumes were recorded at 15 min intervals and expressed as percentages of the amount of water administered. The urine collection was continued over a period of 2 hr.

General procedure Two series of rats were investigated (a) rats fed on a dry diet, without any supply of water, (b) rats which were allowed a free supply of water, but no food. No experiment lasted more than 6 days. During the period of observation rats were kept in individual metabolism cages. Urine was collected under paraffin, and the contamination of urine by faeces was avoided by using glass separators. At the end of the period of observation, the animals were either killed under ether anaesthesia, and blood and tissue samples were taken and analyzed, or they were given a standard amount of water to drink and killed after their water diuresis had been followed for 2 hr.

Statistical treatment Results are given as means and standard error. Student's 't' test (Fisher, 1944) for small samples was used for estimating the significance of means. The probability P for t was obtained from the Tables of Fisher & Yates (1943).

RESULTS

Rats without food, but with free access to water

The intake of water and the urinary excretion were measured daily. The amount of water drunk varied from day to day and from animal to animal, but in spite of important individual variations (Tables 1 and 3), at the end of the 6 days of observation, the

average amount of water drunk over 24 hr had fallen from 4.5 ml/100 g body weight in the controls to 3.3 ml/100 g body weight. This finding agrees with that of Adolph (1947).

Table 1 shows a typical experiment on two rats starved for 6 days. It will be noted that the amounts of water drunk and the amounts of urine excreted were not only irregular, but did not seem to bear any relation to each other. It will also be noted that in spite of the water drunk, the urine remained more concentrated than in the controls where the sp gr was found to be 1.017 ± 0.0016 (Heller, 1949). Towards the end of the experiment there was a fall in the urinary concentration of sodium, chloride and potassium, but not in that of nitrogen.

After 3 days without food, but with free access to water, the plasma protein concentration and plasma water content, as well as the concentration of chloride, sodium, and potassium in plasma, were comparable with those of controls. The total muscle water content was normal, but the concentrations of chloride and sodium in muscle were increased ($t = 4.761$, $P < 0.001$ and $t = 2.020$, $P < 0.1 > 0.05$ respectively), this resulted in an increase of the extracellular fluid phase, it amounted to 20.3 ± 1.41 ml/100 g fat-free tissue, as compared with 16.7 ± 0.50 ml/100 g in controls ($t = 2.978$, $P < 0.01$). Chloride and sodium space, however, remained comparable in size ($t = 0.585$, $P > 0.5$), as in normal animals. Concurrent with the increase of the extracellular fluid phase of muscle, there was a decrease of the intracellular fluid phase ($t = 2.403$, $P < 0.05$), though its concentration of water remained unchanged (Table 2).

After 6 days with water, but without food, the plasma concentrations of proteins, chloride, and sodium were still in the normal range (Table 2), but there was an increase in the plasma concentration of potassium as compared with that of

Table 1 *The effect of withdrawing food from rats with free access to water on body weight and volume and composition of urine*

Rat no	Day	Wt of rat (g)	Water drunk (ml./100 g/24 hr)	Urine excreted (ml./100 g/24 hr)	Urine (sp gr)	Urine (g/100 ml)			
						Cl ⁻	Na	K	N
23	1	272	—	—	—	—	—	—	—
	2	268	2.61	1.86	1.049	0.290	0.150	1.056	0.55
	3	246	0.00	2.00	1.047	0.272	0.131	1.008	0.56
	4	231	3.00	2.16	1.046	0.187	0.110	1.420	0.75
	5	228	4.02	1.91	1.040	0.049	0.043	0.900	—
	6	220	5.45	2.70	1.040	0.045	0.045	1.100	0.54
	7	210	2.86	1.52	1.038	0.101	0.079	0.718	0.59
24	1	355	—	—	—	—	—	—	—
	2	340	0.88	1.76	1.056	0.309	0.196	1.030	0.80
	3	315	0.00	2.06	1.057	0.256	0.158	1.230	0.64
	4	297	1.68	1.69	1.051	0.320	0.125	1.600	0.77
	5	286	1.71	2.45	1.046	0.129	0.122	1.520	1.25
	6	262	1.14	3.00	1.049	0.142	0.114	0.940	0.82
	7	245	0.81	2.98	1.043	0.187	0.100	0.703	0.53

Table 2 *Effects of dehydration and of inanition on the composition of plasma, liver and muscles in rats*

(Number of rats indicated in brackets)

	Plasma					Muscle					Liver		
	Water content (%)	Proteins (g/100 ml)	Cl ⁻ (m equiv/l plasma water)	Na (m equiv/l plasma water)	K (m equiv/l plasma water)	Water content (%)	Cl ⁻ (m equiv/l muscle water)	Na (m equiv/l muscle water)	K (m equiv/l muscle water)	Extracellular fluid phase (ml/100 g)	Intracellular fluid phase (ml/100 g)	Water content/100 g intracellular fluid phase	Intracellular K (m equiv/kg muscle)
Normal rats	91.4 ± 0.13 (27)	7.0 ± 0.09 (27)	93.2 ± 2.34 (27)	135.3 ± 7.05 (18)	5.6 ± 0.29 (17)	71.4 ± 0.31 (27)							
Rats deprived of food but with free access to water													
(a) For 3 days	91.8 ± 0.41 (9)	7.1 ± 0.21 (9)	95.1 ± 4.68 (9)	142.1 ± 5.92 (9)	5.5 ± 0.18 (9)	71.0 ± 0.49 (9)							
(b) For 6 days	92.1 ± 0.14 (12)	7.0 ± 0.17 (11)	93.1 ± 4.24 (12)	141.7 ± 2.64 (12)	6.4 ± 0.18 (12)	72.0 ± 0.54 (12)							
Rats allowed dry food but no water for 6 days	91.1 ± 0.10 (12)	7.6 ± 0.08 (12)	103.1 ± 3.24 (12)	156.9 ± 6.83 (12)	10.0 ± 0.53 (12)	71.6 ± 0.39 (12)							
Rats with free access to dry food for 5 days, and allowed water on the 6th day	92.1 ± 0.04 (9)	7.2 ± 0.07 (9)	84.5 ± 1.91 (9)	104.3 ± 0.75 (9)	5.8 ± 0.22 (9)	75.0 ± 0.53 (9)							
Normal rats	75.7 ± 0.32 (27)	20.2 ± 0.47 (27)	28.8 ± 1.48 (17)	130.2 ± 7.73 (17)	16.7 ± 0.50 (27)	59.3 ± 0.50 (27)							
Rats deprived of food but with free access to water													
(a) For 3 days	76.5 ± 0.11 (9)	25.2 ± 1.06 (9)	33.7 ± 3.20 (9)	113.3 ± 4.50 (9)	20.3 ± 1.41 (9)	56.2 ± 1.44 (9)							
(b) For 6 days	76.7 ± 0.44 (12)	28.0 ± 1.56 (12)	47.5 ± 3.09 (12)	110.6 ± 2.60 (12)	22.3 ± 3.40 (12)	54.8 ± 1.14 (12)							
Rats allowed dry food but no water for 6 days	73.3 ± 0.20 (12)	23.2 ± 1.96 (12)	42.6 ± 7.64 (12)	117.4 ± 3.20 (12)	15.6 ± 0.31 (12)	57.5 ± 0.53 (12)							
Rats with free access to dry food for 5 days, and allowed water on the 6th day	78.7 ± 0.35 (9)	27.1 ± 1.88 (9)	41.5 ± 2.25 (9)	117.0 ± 8.83 (9)	22.4 ± 1.85 (9)	56.3 ± 1.70 (9)							

controls ($t=2.045$, $P=0.05$) The total amount of muscle water was not significantly different from that of normal animals, but the extracellular fluid phase, expressed in terms of chloride space, was markedly increased, it amounted to 22.3 ± 3.40 ml/100 g fat-free tissue instead of 16.7 ml/100 g in controls. In contrast with normal rats, in which chloride and sodium occupied a comparable space (Dicker, 1948), the sodium space in this series of rats was greater than that of chloride ($t=2.028$, $P<0.1>0.05$), indicating that sodium had penetrated into the muscle cells. The amount of muscle potassium decreased significantly as compared with controls ($t=3.023$, $P<0.01$).

There was thus clear evidence of tissue oedema in the starved rats with access to water. It will be noted, however, that the standard error of the calculated mean value for the extracellular fluid phase was much greater than that of controls (Table 2) the coefficient of variation of the extracellular fluid phase in the series of rats starved for 6 days amounted to $52.8 \pm 10.80\%$, as compared with $15.0 \pm 2.04\%$ in normal rats (standard error

of difference = 3.44). This significant increase of the coefficient of variation was correlated with the variability in the amount of water drunk by these rats. Table 3 shows changes in the body weight of two rats compared with the amount of water drunk and that of urine excreted per 100 g/24 hr during 6 days of starvation. It will be seen that the amounts of urine/100 g body weight/24 hr were comparable, but that the amounts of water drunk were very different they averaged 3.1 ml/100 g/24 hr in one rat, and only 0.6 ml/100 g/24 hr in the other. This resulted in a discrepancy in the decrease of body weights. The loss of body weight amounted to 24.8 and 33.0% respectively. Concurrently with the discrepancy in the water load, the plasma ionic concentration, the muscle water content and the extracellular fluid phases of these two rats were markedly different (Table 3). These differences suggested that there was a failure in the mechanism of water excretion. To investigate this hypothesis, a standard amount of water was administered to rats which, though starved, had free access to water. Table 4 shows the renal response to water.

Table 3 *The effect of withdrawing food from rats with free access to water on body weight, plasma and muscle composition*

(All values for muscle are expressed/100 g fat-free tissue)

Rat no	Day	Decrease in body wt (%)	Water drunk (ml./100 g / 24 hr)	Urine excreted (ml./100 g / 24 hr)	Plasma concentration (m-equiv/l)			Muscle (ml./100 g)		
					Cl ⁻	Na	K	Water content	Extra-cellular fluid phase	Intra-cellular fluid phase
17	1	7.5	Nil	2.2	112.7	146.5	6.3	75.6	17.3	58.3
	2	6.5	1.0	1.8						
	3	6.0	0.8	2.3						
	4	6.0	0.7	2.0						
	5	7.0	0.5	2.7						
	6	6.5	0.6	2.3						
18	1	8.2	Nil	2.2	92.4	130.0	5.1	77.0	23.7	53.3
	2	6.1	3.0	2.1						
	3	2.0	3.9	2.0						
	4	3.5	5.5	2.7						
	5	3.0	3.0	1.5						
	6	3.0	4.3	2.1						

Table 4 *Water diuresis in starved and dehydrated rats*

(Each dose of water was 5% of the body wt and was administered by stomach tube)

Time after water administration (min)	Urine output (% of administered dose)					
	60 normal rats	10 starved rats with water <i>ad lib</i>		12 rats dehydrated for 6 days		
		After 3 days	After 6 days	First dose of water	Second dose of water	Third dose of water
15	Nil	Nil	Nil	Nil	Nil	Nil
30	1.9 ± 0.50	Nil	Nil	Nil	Nil	Nil
45	10.0 ± 0.82	1.9 ± 0.71	"	"	"	"
60	22.4 ± 1.54	7.4 ± 2.59	6.4 ± 1.31	"	"	"
75	37.0 ± 1.83	17.4 ± 4.00	15.2 ± 2.45	"	"	8.0 ± 2.53
90	52.4 ± 2.00	29.0 ± 7.02	22.1 ± 3.55	"	"	16.0 ± 4.36
105	69.4 ± 1.81	37.9 ± 7.13	30.3 ± 5.70	"	"	19.0 ± 5.02
120	81.9 ± 0.94	44.0 ± 8.24	37.5 ± 7.09	"	"	26.0 ± 5.20
						30.0 ± 7.57

administration in rats starved for 3 and 6 days after only 3 days of starvation, and in spite of the fact that the animals had water *ad lib*, there was a delay in the onset of the diuresis, and a very marked decrease of the urinary volume. Ninety minutes after water administration the amount of urine excreted amounted to $29.0 \pm 7.02\%$ of the water given as compared with $52.4 \pm 2.00\%$ in controls (Table 4).

This finding and the fact that the samples of urine excreted during the period of experimentation had a relatively high specific gravity (Table 1) suggest that the water retention, and hence the tissue oedema might be, directly or indirectly, of renal origin.

Rats fed on a dry diet, without access to water

During the first 24 hr the intake of food was normal, i.e. between 15 and 20 g/animal, yielding between 50 and 60 cal/day, which compared well with controls, but from the second day, the amount of food eaten fell sharply, and from the third to the sixth day all the animals refused to eat. During the last 2 days the rats became extremely nervous and restless, biting the wiring of their cage and trying to escape.

The urinary volume excreted/24 hr decreased progressively from the first to the sixth day. Table 5 shows a typical experiment on two rats. The analyses of the urine samples reported in Table 5 are those of the second, fourth and sixth days. The most striking feature was the disappearance of chloride in the urine, in spite of the fact that the specific gravity remained high (Table 6). This disappearance of urinary chloride cannot be explained by a decrease in the concentration of plasma chloride (Table 5), nor is it the result of a failure in the ability of the kidney to concentrate (Tables 5 and 6). However, from the fact that on the sixth day the concentrations of chloride, sodium, potassium and urea were higher in the plasma of these rats than in controls, it may be assumed that the glomerular filtration rate of the kidneys was decreased.

After 6 days without water, the plasma water content of rats amounted to $91.1 \pm 0.10\%$ (Table 2), and their chloride, sodium and potassium concentrations were significantly higher than in controls ($t = 7.630, P < 0.001, t = 2.037, P = 0.05$ and $t = 6.738, P < 0.001$, respectively). The total water content of muscles in this series of thirsting rats amounted to 73.5 ml/100 g fat-free tissue, which was

Table 5 *Effects of withdrawal of water on the composition of urine and plasma of rats*

(On the second, third and fourth days the urine was collected over a period of 24 hr)

Day of observation		Vol (ml)	Cl ⁻ (g/100 ml)	Na (g/100 ml)	K (g/100 ml)	NH ₃ (g/100 ml)	Urea (g/100 ml)
Male rat (initial wt 298.5 g, final wt 260.0 g)							
Second	Urine	6.65	0.283	0.332	1.023	0.889	2.705
Fourth	Urine	2.90	0.064	0.115	1.421	2.370	9.350
Sixth	Urine	1.80	0.011	0.086	1.591	5.785	9.075
Sixth	Plasma	—	0.327	0.318	0.028	—	0.108
Male rat (initial wt 272.5 g, final wt 190.0 g)							
Second	Urine	4.5	0.476	0.290	0.984	1.036	2.857
Fourth	Urine	2.6	0.429	0.447	1.635	1.580	11.475
Sixth	Urine	2.5	0.015	0.190	1.787	4.350	8.325
Sixth	Plasma	—	0.312	0.307	0.030	—	0.129

Table 6 *Effects of dehydration and of subsequent administration of water on the body weight and volume and composition of urine of rats*

(The rats were deprived of water from day 1 to day 6, and free access to water was allowed on day 7)

Day	Average body wt (g)	Average wt of food eaten (g)	Average vol of water drunk (ml)	Urine				
				Average vol excreted (ml/24 hr)	Sp gr	Cl ⁻ (g/100 ml)	Na (g/100 ml)	K (g/100 ml)
1	200	—	—	—	—	—	—	—
2	181	19.0	Nil	8.8	1.060	0.390	0.304	0.749
3	171	6.5	"	2.5	1.060	0.224	0.201	0.806
4	159	Nil	"	2.6	1.060	0.171	0.103	0.826
5	154	"	"	1.2	1.060	0.045	0.036	0.944
6	149	"	"	0.8	1.059	0.043	0.020	0.713
7	164	"	21.0	3.5	1.042	0.021	0.017	0.720

significantly lower than that of controls ($t=3.818$, $P<0.001$). The loss of muscle water affected both the extracellular and the intracellular fluid phases, but not in the same manner. In spite of an increase in its ionic concentration (Table 2), the extracellular fluid phase, expressed in terms of chloride space, decreased from an average of 16.7 to 15.6 ml/100 g fat free tissue ($t=2.026$, $P<0.05$). The hypertonicity of the extracellular fluid was checked by the concurrent loss of water from the intracellular fluid phase, which fell from an average of 59.3 ml in control animals to 57.5 ml/100 g fat free tissue ($t=2.015$, $P=0.05$). The magnitude of this water loss can be best estimated by comparing the concentration of water of the intracellular phase in the present series of rats with that of normal rats, in normal animals, the intracellular water concentration amounted to 71.2 ml/100 g but was only 68.2 ml/100 g in dehydrated rats ($t=4.225$, $P<0.001$). The concentration of intracellular potassium followed that of water and decreased from 97.2 to 87.3 m-equiv/kg muscle (Table 2). It is thus likely that while the extracellular fluid phase constituted the first line of defence against dehydration, intracellular water must have been made available to avoid an increase in the ionic concentration of the body fluid which would have been fatal to the animal.

The extent of the state of dehydration in this series of rats could be indirectly assessed by observing the amount of water needed to induce a water diuresis. Table 4 shows that in a series of 12 rats, which had been dehydrated for 6 days, the administration of 5% of their body weight of water failed to produce a water diuresis. Three hours later a second administration of the same amount of water also failed. A third administration of the standard amount of water, however, produced a moderate urinary excretion.

This finding led to the question. Was all that water (15% of their body weight) used to reduce the increased osmotic pressure of the body fluids, or was its retention in the tissues partly the result of the failure of the kidneys to excrete it? In the following experiment a group of six rats, each weighing about 200 g, was kept in a metabolism cage for 6 days. During the first 5 days they were given dry food but no water, on the sixth day food was withheld but water allowed. During the first 24 hr of observation, they ate an average of 19.0 g of dry food/rat, on the second day, the average quantity of food eaten amounted to 6.5 g only, from then on, all the animals refused to eat (Table 6). The urine was collected daily. Table 6 shows the average volume of urine excreted/24 hr/animal, its specific gravity and the concentration of chloride, sodium and potassium, it gives also the average amount of water drunk/rat during the last

24 hr. In spite of an average amount of 21.0 ml being drunk (representing 13% of the body weight) the average urinary volume excreted did not exceed 3.5 ml, i.e. 2.1% of the body weight (Table 6). Furthermore, there were no signs of increased excretion of chloride, sodium or potassium. The sudden increase of the average body weight indicated clearly that most of the water drunk had been retained by the tissues (Table 6). When killed, a gross post-mortem examination showed that the intestinal tract was oedematous, and that the muscles and liver were abnormally 'wet'.

Further investigations showed that the plasma water content and plasma protein concentration were normal (Table 2), but that the plasma concentration of chloride and of sodium were significantly decreased when compared with normal ($t=2.060$, $P<0.05$ and $t=2.988$, $P<0.01$), while the plasma concentration of potassium had returned to normal values (Table 2).

The total water content of liver and of muscle was markedly increased and exceeded significantly that of normal rats ($t=5.164$, $P<0.001$ and $t=6.533$, $P<0.001$). Examining the partition of the water in muscle, it could be shown (Table 2) that the increase of the total muscle water was mainly the result of an increase of its extracellular fluid phase. The intracellular fluid phase, however, remained comparable to that of dehydrated animals, though its concentration of water returned to normal values (Table 2). It can thus be concluded that the increased osmotic pressure of the body fluid in dehydrated rats was not the only factor opposing further loss of body water, when these animals were allowed water, the amount of water drunk exceeded that required to bring the enhanced ionic concentration back to normal values. As this excess of water load was not excreted by the kidneys it resulted in a marked tissue oedema. It is, therefore, likely that during dehydration of animals the renal mechanism of water excretion intervenes directly or indirectly in the preservation of body water.

DISCUSSION

In the two series of rats investigated, those which were starved but had ample supplies of water, and those allowed a dry diet but no water, the first developed an early tissue oedema, the others did not. Though some food was eaten during the 2 first days, the rats of the second series soon refused to eat and starved for the last 3-4 days of the experiment. It may, therefore, be assumed that both series suffered from a comparable degree of starvation, but that they differed in that one was allowed access to water and the other not.

According to accepted theories, the mechanism of water preservation in dehydrated animals can be

represented as follows. At the beginning, the extracellular fluid phase is kept normal by transfer of water made available from the intracellular phase, where it has been released by the consumption of protoplasm incidental to fasting (Gamble, 1947). This shift of water to the extracellular fluid phase is accompanied by an extrusion of the intracellular base, potassium (Elkinton & Winkler, 1944, Heller, 1949). As the dehydration proceeds, water is drained from the extracellular fluid phase, with the result that the latter tends to become hypertonic (Elkinton & Taffel, 1942, Winkler, Elkinton, Hopper & Hoff, 1944). This in turn produces more transfer of water from the intracellular into the extracellular fluid phase. The maintenance of the extracellular fluid phase, within limits compatible with the survival of the organism, is thus ultimately provided at the expense of the intracellular phase (Table 2). This interpretation, derived from the estimation of body fluid lost, and calculated from changes in body weight and from the urinary excretion of sodium, potassium and nitrogen, is based entirely on the assumption that the renal function remains normal.

It has repeatedly been demonstrated that during advanced dehydration there is a functional renal failure (see McCance, 1936) leading to retention of crystalloids. The results of the present series of experiments show that dehydration in rats produced ultimately a retention of urea, potassium, chloride and sodium in the blood, suggesting impairment of renal function. Furthermore, Gilman & Goodman (1937) have shown that the urine of dehydrated rats contains significant amounts of an antidiuretic substance, which they assumed to be similar to 'vasopressin'. It seems likely, therefore, that the attempt to explain the shifts of water during dehydration by a purely physical mechanism, like osmotic pressure, omits one important factor, viz. the influence of the kidney.

This hypothesis of a renal intervention is supported by the results of the present experiments where water was allowed to rats which had been so deprived for 5 days. Adolph (1947) claimed that, when water was again offered to rats which had been entirely deprived of it for several days, only a 'small excess' of water was ingested. In contrast with these findings it could be shown that the amount of water drunk in 24 hr. by rats which had been previously dehydrated for 5 days amounted to 13% of their body weight. (Controls drink on the average 4.5% of their body weight of water in 24 hr.) The corresponding urinary volume, however, amounted only to just over 2% of their body weight as against 5% in normals. The marked discrepancy between the amount of water drunk and that of urine excreted resulted in a sudden increase of body weight of nearly 10% (Table 6). Further-

more, it could be shown that the total water content of the liver and muscle of the test rats exceeded significantly that of normal rats, and that there was a marked increase of the extracellular fluid of muscle, i.e. clear symptoms of tissue oedema (Table 2).

Had the osmotic pressure been the only factor responsible for the regulation of the volume of body fluid in dehydrated animals, it would be difficult to understand why the regulation failed when the rats were allowed to drink. Besides the mechanism of hypertonicity, there must have been one which opposed the renal excretion of the excess water load. From the results presented there is evidence that, both the rate of glomerular filtration, and that of tubular water reabsorption were affected. It was, however, outside the scope of this work to determine the factor that produced both a decrease of the rate of glomerular filtration and an increase of that of tubular water reabsorption.

In contrast with dehydrated animals, rats which were starved, but allowed free access to water, developed signs of tissue oedema. As early as 3 days after the beginning of the experiment, the chloride space of muscle was significantly greater than that of normal rats (Table 2). The objection might be raised that to equate chloride space with extracellular fluid phase holds only for normal rats, where chloride and sodium occupy a comparable volume of distribution (Dioker, 1948), and that in starved animals there might be changes in the cell permeability which would account for changes in the chloride distribution. However, the fact that chloride occupied a comparable fraction of the muscle water in normal and starved rats suggests that chloride space can be assumed to give some measure of the extracellular fluid phase in muscle. It must be remembered, however, that chloride space is likely to be somewhat larger than the true value of the extracellular space, even in normal animals (Fulton, 1947).

As these rats were deprived of food, it could be assumed that the decrease of the intracellular fluid phase observed in this series (Table 2) was the result of a loss of water, released from the cells as a consequence of the degradation of proteins incidental to fasting. This loss of intracellular water was accompanied by a loss of cell potassium, which proceeded in spite of the fact that these animals could, by drinking, maintain an apparently normal state of hydration. Furthermore the loss of intracellular water seemed to be independent of the amount drunk.

The extracellular fluid phase, on the other hand, was not only increased, but the magnitude of the increase was directly correlated with the amount of water drunk (Table 3). This suggests at once a failure in the regulation of water excretion in these

rats, as is also indicated by the following facts (a) the urinary volume did not bear any relation to the amount of water drunk, (b) in spite of the fact that water was freely obtainable, the specific gravity of the urines remained much higher than in normal rats which had free access to food and water, (c) following the administration of a standard amount of water, the onset of urinary excretion was delayed and its total volume diminished

Comparing the results obtained in starved rats having free access to water with those in rats without water the following conclusions could be reached (a) prolonged starvation, up to 6 days, leads to tissue oedema when water is drunk, (b) in starved animals, allowed free access to water, there is a direct relation between the amount of water drunk and the magnitude of the extracellular fluid phase, expressed in terms of chloride space, (c) the onset of tissue oedema in starved rats with access to water does not bear any relation to the plasma protein concentration, and seems mainly to be the result of a failure in the mechanism of water excretion

SUMMARY

1 Total water content of plasma, muscle and liver and the chloride and the sodium space of muscle were estimated in two series of rats (a) those kept for 3 or 6 days without food but with free access

to water, (b) those allowed dry food but no water for 6 days

2 Group (a) developed an early tissue oedema, and after only 3 days the extracellular fluid phase of their muscle, expressed in terms of chloride space, was significantly increased. When water equal to 5% of their body weight was administered, the onset of the water diuresis was delayed, and the volume of urine excreted in 2 hr was lower than in normal rats

3 Rats allowed a dry diet but no water for 6 days (group (b)) showed retention of chloride, sodium, potassium and urea in the plasma, accompanied by a decrease of the intracellular and the extracellular fluid phase of the muscles. Administration of water (as above) failed to produce a water diuresis. When offered water *ad lib* after 5 days of water deprivation, an amount equal to 13% of their body weight was drunk and the animals developed tissue oedema, the total water content of the liver and muscle was significantly increased over that of normal animals, and so was the extracellular fluid phase of muscle. The plasma protein concentration, however, remained normal

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Colorimetric Determination of Potassium by Folin-Ciocalteu Phenol Reagent

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A method for the colorimetric estimation of manganese by reduction of the phosphomolybdic phosphotungstic acid phenol reagent has been described (Abul-Fadl, 1948). Cobalt was also found to give a similar colour reaction in the presence (but not in the absence) of amino acids. Conversion of cobalt into the cobaltinitrite did not affect its response to this reaction.

As methods for the estimation of potassium in biological fluids depend on precipitation as cobaltinitrite, and subsequent estimation of one of the constituents of the precipitate, it was thought that this colour reaction might be useful in this connection.

Amongst colour reactions hitherto used for the microdetermination of potassium are those of Doisy & Bell (see Briggs, 1923), and of Looney & Dyer (1942). Both reactions are based on the formation of an azo compound by the nitrite radicals of cobaltinitrite. Theoretically, those methods which determine directly a stable constituent of the precipitate, e.g. cobalt, are preferable to those which depend upon the unstable nitrite radical. Breh & Gaebler (1930) described a method in which cobalt is determined as the thiocyanate. This method involves the precipitation of potassium as the silver cobaltinitrite complex which is more sparingly soluble than the customary $K_2NaCo(NO_2)_6$. The method is, however, more troublesome to execute and the colour reaction involved is not entirely satisfactory, since it depends upon such factors as temperature, ethanol concentration, etc. Jacobs & Hoffman (1931) introduced a reaction between alkaline ferrocyanide and cobalt compounds in the presence of choline hydrochloride, which gives a stable green colour.

EXPERIMENTAL

Solutions

Sodium cobaltinitrite reagent (Kramer & Tisdall, 1921) As described by King (1947).

Standard potassium solution K_2SO_4 (0.2228 g A.R.) is dissolved in 500 ml of water, giving a solution which contains 20 mg K/100 ml.

Glycine (1M) 7.5 g/100 ml water. The solution is filtered and preserved with a few drops of chloroform.

Sodium carbonate solution (25% w/v) Anhydrous Na_2CO_3 (25 g) is dissolved in warm water and made to 100 ml. This solution is kept in a warm place.

Phenol reagent of Folin & Ciocalteu (1927) For method of preparation see King (1947). This reagent is diluted for use (1 vol reagent + 2 vols water).

Method

Principle Alkaline solutions of cobalt salts, in presence of a trace of amino acid (glycine or alanine), reduce the phosphomolybdic phosphotungstic acid phenol reagent to a blue colour, the intensity of which is directly proportional to the amount of cobalt present, and hence, if potassium has been precipitated as cobaltinitrite, to the amount of potassium in the original solution.

Procedure The precipitation of potassium from serum is carried out in principle according to the method of Kramer & Tisdall (1921) as adapted by King (1947). In a 15 ml conical centrifuge tube, marked at 6 ml, is placed 0.2 ml of serum.

In a similar tube is placed 0.2 ml of standard potassium solution (containing 20 mg K/100 ml). To each tube is slowly added 0.5 ml of filtered sodium cobaltinitrite reagent with constant shaking. After 45 min 1 ml of water is added, and the contents are mixed and centrifuged at moderate speed for 15 min. The tubes are then inverted and briefly drained on filter paper, 2 ml of water are added down the side of each tube without disturbing the precipitate. The tubes are again centrifuged for 5 min, inverted and thoroughly drained. The precipitates are washed with 5 ml 70% ethanol, which is blown into the tubes so as to stir up the precipitates. After centrifuging and draining thoroughly, 2 ml of water are added to each tube, and the tubes placed in a boiling water bath until dissolution is complete. A third tube containing 2 ml distilled water is used as a blank.

To each of the three tubes, while still hot, 1 ml glycine solution (7.5%) and 1 ml Na_2CO_3 solution (25%) are added and thoroughly mixed. 1 ml diluted Folin Ciocalteu phenol reagent is then added to each, the contents are mixed again, and the tubes are allowed to stand in a water bath at 37° for 10–15 min. After cooling to room temperature the volume is accurately adjusted to 6 ml in each tube, and the colours are read in a photoelectric colorimeter, using a red filter and setting the zero with the blank. The colours are stable for several hours.

RESULTS

The effect of glycine on the blue colour reaction is shown in Fig. 1. Reduction of the phosphotungstic-phosphomolybdic acids by cobalt salts could not

be effected in the absence of amino acids. As little as 0.005 M-glycine is enough to develop the colour reaction in solutions containing 0.01 mg Co. The colour, however, is rapidly and optimally obtained in the presence of 0.2–0.5 M-glycine. Higher concentrations, on the other hand, reduce the colour intensity. Other amino-acids are also effective, although those of higher molecular weight than alanine give with the phenol reagent in the absence of cobalt blue colour reactions, the intensity of which increases with the rise in their molecular weight.



Fig 1 Effect of glycine concentration on blue colour development in the estimation of $55\mu\text{g}$ K as cobalt nitrite by Folin Ciocalteu reagent

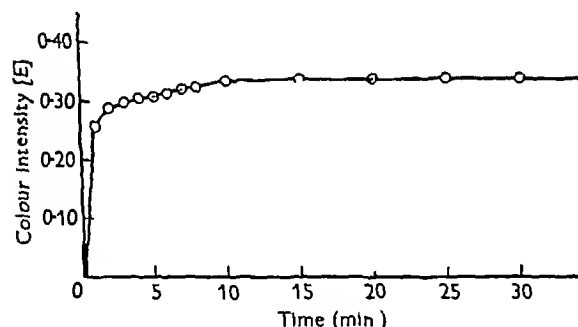


Fig 2 Rate of Folin Ciocalteu blue colour development at room temperature $50\mu\text{g}$ K in 0.2 M glycine

Fig 2 shows the rate of the blue colour development at room temperature in 0.2 M-glycine. The colour attains its maximum intensity in about 15–20 min at room temperature, but much more rapidly at 37° . The blue colour, however, once formed, is stable for several hours and only starts to fade very slowly after 24 hr.

A strict correlation between the intensity of the blue colour and the quantity of potassium present as cobalt-nitrite complex is shown in Fig 3. The relative intensities of the blue colour obtained by the new method and of the green colour of the choline ferrocyanide are shown on the same figure. It is evident that the new colour reaction provides an accurate and more sensitive method for estimation of potassium.

This is further illustrated in Tables 1 and 2 which give results of potassium determinations in normal and pathological sera by the new method, by that of Jacobs & Hoffman (1931) and by the flame

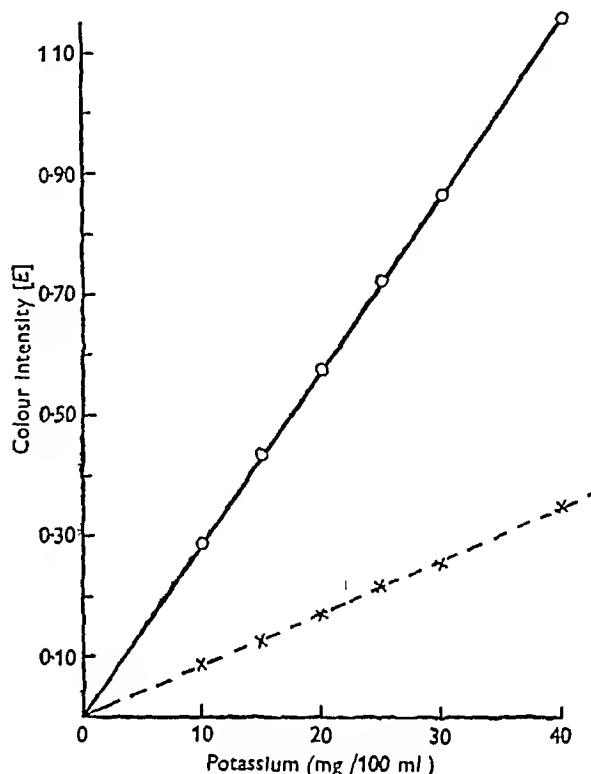


Fig 3 Colorimetric estimation of potassium in 0.5 ml potassium sulphate standard solutions by the new (Folin Ciocalteu) method and by that of Jacobs & Hoffman (1931). The colour intensities were measured in a Hilger Spekker absorptiometer. — Folin Ciocalteu blue colour (Ilford spectrum red filter, no 608), - - - choline ferrocyanide green colour (Ilford spectrum orange filter, no 607)

photometer (cf Klyne, 1948). Table 3 gives the results of analyses carried out by the new method and by the flame photometer on a serum to which known amounts of potassium had been added.

DISCUSSION

The method described above suffers from the disadvantage, common to all other methods involving the indirect estimation of potassium by precipitation in different complex forms and estimating some constituent other than potassium, viz that the complex may vary in composition according to conditions of precipitation. These methods, however, are still in general use, although the flame photometric methods may take their place (Barnes, Richardson, Berry & Hood, 1945; Hald, 1947; Domingo, Klyne & Weedon, 1948; Klyne, 1948).

The method, nevertheless, offers a simple and accurate means for the micro estimation of potassium in biological fluids. The determination has

Table 1 *Determination of serum potassium by different methods*

Serum			Results			
			Folin Ciocalteu method		Jacobs & Hoffman method	
			Photometric readings* (E)	Found (mg K/100 ml serum)	Photometric readings* (E)	Found (mg K/100 ml serum)
1	Normal	0.2	0.270	20.0	—	—
		0.2	0.265	19.7	—	—
		0.5	0.685	20.3	0.175	20.0
		0.5	0.690	20.4	0.180	20.3
2	Normal	0.2	0.245	18.0	—	—
		0.5	—	—	0.160	18.2
3	Acute nephritis	0.2	0.400	29.6	—	—
		0.5	1.000	29.4	0.258	29.4
		0.5	—	—	0.265	30.2
4	Periarteritis nodosa	0.2	0.370	27.5	—	—
		0.2	0.375	27.7	—	—
		0.5	0.950	27.9	0.247	27.5
		0.5	0.960	28.2	—	—
5	Alkalosis	0.2	0.310	22.8	—	—
		0.2	0.310	22.8	—	—
		0.5	—	—	0.205	22.8
6	Acute nephritis	0.2	0.295	21.9	—	—
		0.2	0.300	22.2	—	—
		0.5	0.770	22.6	0.190	21.1
		0.5	0.760	22.4	—	—
7	Arthritis	0.2	0.300	22.2	—	—
		0.2	0.295	21.8	—	—
		0.5	0.740	21.8	0.200	22.2
		0.5	0.750	22.1	—	—
8	Addison's disease	0.2	0.255	19.0	—	—
		0.2	0.260	19.2	—	—
		0.5	0.660	19.4	0.170	19.4
9	Meningitis	0.2	0.275	20.5	—	—
		0.2	0.280	20.7	—	—
		0.5	0.700	20.6	0.190	21.1
		0.5	0.708	20.8	—	—
10	Nephritis	0.2	0.290	21.5	—	—
		0.5	0.730	21.4	0.187	21.0
11	Alkalosis	0.2	0.270	20.0	—	—
		0.2	0.270	20.0	0.175	19.7
12	Nephritis	0.2	0.250	18.5	—	—
		0.5	0.610	17.8	0.150	17.3

* Ilford tricolour red light filter

Table 2 *Determination of potassium in normal sera by the new Folin-Ciocalteu method and the flame photometer*

No. of specimens examined	22
	K (mg/100 ml)
Average value by new method	17.6
Range	15.5-21.0
Average value by flame photometer	17.7
Range	15.3-21.4
Root mean square difference between the two methods	1.7

been conveniently carried out on 0.2 ml of serum instead of 0.5 ml, the minimum required for Jacobs & Hoffman's (1931) method. It has been successfully applied to routine work.

Table 3 *Recoveries of potassium added to normal serum*

Potassium added to serum (mg/100 ml)	Total potassium found (mg/100 ml)	
	New method*	Flame photometer†
0	19.5 19.0	19.7
5	25.7 24.9	24.1
10	32.0 30.0	29.0
15	34.2 34.2	34.9
20	39.0 40.0	39.7

* 0.2 ml serum used

† 1.0 ml serum used

SUMMARY

1 A micromethod is described for the estimation of potassium in sera and biological fluids

2 The method depends on the precipitation of potassium as cobaltinitrite, and the colorimetric estimation of the cobalt in the latter by the re-

duction of the Folin-Ciocalteu phosphomolybdic-phosphotungstic phenol reagent

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The Nicotinamide-Saving Action of Tryptophan and the Biosynthesis of Nicotinamide by the Intestinal Flora of the Rat

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Tryptophan can replace nicotinamide in the diet of the rat (Krehl, Sarma, Teply & Elvehjem, 1946, Singal, Sydenstricker & Littlejohn, 1947a), the dog (Singal, Sydenstricker & Littlejohn, 1947b), the pig (Luecke, McMillen, Thorp & Tull, 1947) and the rabbit (Wooley, 1947). It increases the urinary elimination of nicotinamide metabolites in man (Perlzweig, Rosen, Levitas & Robinson, 1947, Sarett & Goldsmith, 1947), dog (Singal *et al.* 1947b), rat (Rosen, Huff & Perlzweig, 1946, Singal, Briggs, Sydenstricker & Littlejohn, 1946, Schweigert & Pearson, 1947), mouse (Schweigert & Pearson, 1947), cotton rat (Schweigert, Pearson & Wilkening, 1947) and horse (Schweigert *et al.* 1947). The mechanism of the replacement of nicotinamide by tryptophan is obscure. The fact that tryptophan increases the elimination of nicotinamide metabolites favours the conception of a direct conversion. However, kynurenine acid (Ellinger, 1904), kynurenine (Kotake, 1931) and xanthurenine acid (Lepkowsky, Roboz & Haagen-Smit, 1943) are the only known end products of tryptophan metabolism in mammals. Kynurenine acid has been shown unable to replace nicotinamide (Rosen, Huff & Perlzweig, 1947) and being an α substituted pyridine derivative cannot be directly converted into nicotinic acid. Kynurenine and xanthurenine acid are found in the urine

* Some of the results presented in this paper have been briefly reported (Ellinger & Abdel Kader, 1947, 1948)

of mammals mainly in pyridoxin deficiency. Beadle, Mitchell & Nyc (1947) believe they have shown that nicotinic acid can be formed from kynurenine by a mutant strain of *Neurospora*. Biosynthesis of nicotinamide is known to occur in numerous mammals and might take place either in the mammalian tissues or in the intestinal tract by the activity of the intestinal flora. It is of interest to study the mechanism of the tryptophan conversion, to find out whether the intestinal bacteria are involved in this phenomenon, and if so to find the way in which the conversion takes place. Participation of the intestinal flora is indicated by the work of Schweigert & Pearson (1947), who showed that the increase of nicotinamide methochloride elimination is more marked after oral than after parenteral administration of tryptophan to rats.

Biosynthesis of trigonelline in plants (*Trigonella foenum graecum*) is stimulated by administration of ornithine, arginine, proline or glutamic acid and further increased by hexamethylene tetramine (Klein & Linser, 1932, 1933a, b). It is possible that one of the above amino-acids might be an intermediate between tryptophan and nicotinamide. Possible pathways from ornithine to nicotinic acid have been suggested by Klein & Linser (1932) and by Guggenheim (1940). In the present paper the role of the intestinal flora in the conversion of tryptophan into nicotinamide was studied in rats *in vivo*,

in vitro experiments were carried out to study the biosynthesis of nicotinamide by intestinal bacteria of the rat from various possible precursors

EXPERIMENTAL

In vivo experiments

Twelve rats of both sexes and two strains (hooded of the Lister stock and albino, own stock of P E) of 200–250 g were kept on a mixed diet (Ellinger, Fraenkel & Abdel Kader, 1947). They were placed in metabolism cages and 24 hr samples of urine were assayed for nicotinamide methochloride at first by the method of Coulson, Ellinger & Holden (1944) and later by that of Huff & Perlzweig (1947). When the nicotinamide methochloride output was found to be constant, each rat was given three doses of 100 mg of DL tryptophan at intervals of 3 or 4 days, alternately orally and parenterally, starting with either oral or parenteral administration. Parenterally administered doses were injected intraperitoneally, and those given orally were ingested from a syringe by stomach tube. The 24 hr nicotinamide methochloride elimination was measured. Another two groups of four rats each of both strains were kept on a similar diet, and the daily nicotinamide methochloride elimination was measured before and after oral administration of 100 mg of DL-tryptophan. They were then given 1 g succinylsulphathiazole with the usual diet. The 24 hr nicotinamide methochloride output was measured again for 3 days, then DL-tryptophan (0.1 g) was given by mouth and the nicotinamide methochloride elimination was again assayed. Three other hooded rats whose nicotinamide methochloride response to orally administered 100 mg tryptophan had been examined, received with their food 0.1 g of sulphathiazole instead of succinylsulphathiazole daily for 4 days. Tryptophan (0.1 g) was then given again by mouth and the nicotinamide methochloride elimination measured. In three hooded rats receiving succinylsulphathiazole and those receiving sulphathiazole the concentration of total and free sulphathiazole in blood was measured by the method of Bratton & Marshall (1939) on the day tryptophan was administered. For this purpose 0.5 ml. of blood was withdrawn by heart puncture. Other rats of 250–300 g body weight similarly fed were given orally and parenterally single doses of L ornithine (0.2 g), L proline (0.2 g) and 4 amino valeric acid (0.5 g) and the daily nicotinamide methochloride output was measured before and after administration of these compounds.

In vitro experiments

In order to obtain cultures from the intestinal flora, rats of the Lister stock which were known to eliminate much nicotinamide methochloride were gassed, and the contents of their caeca were suspended under sterile conditions in 10 ml peptone water. Some samples of this suspension were dried immediately in an ascorbic acid gelatin medium (Stamp, 1947). Part of the undiluted suspension was spread on agar plates which were incubated both aerobically and anaerobically for 48 hr and then examined for types of colonies. The types were isolated and subcultured aerobically and anaerobically. Part of the suspension was treated similarly after heating to 65° for 40 min to examine for spore bearers. Part of the suspension, diluted 100 times

in peptone water, was used to inoculate tubes containing 10 ml of ammonium lactate (Fildes, 1938) or casein hydrolysate (Barton Wright, 1944) media. Amino acids to be tested were added for these media. After incubation at 37° for 24 hr the cultures were autoclaved, and the acid produced was titrated with 0.1 N NaOH using cresol red or bromothymol blue as external indicators. The nicotinamide formed was assayed by the method of Barton Wright (1944). In a similar way tubes, containing either ammonium lactate or casein hydrolysate media, were incubated with all non coliform bacteria isolated from the mixed cultures. The bacteria were grown for 24 hr alone, the contents of the tubes were sterilized by filtration or autoclaving, then inoculated with *Bacterium coli* (*Escherichia coli*), incubated again for 24 hr and assayed as before. In other experiments *Bact. coli* were added simultaneously with the non coliform bacteria, incubated and examined for acid and nicotinamide formation.

The following compounds were tested: L-ornithine, L-arginine, DL-glutamine, DL-glutamic acid, L-citrulline, L-proline, L-leucine, L-isoleucine, DL-valine, DL-tryptophan, 4-aminovaleric acid, DL-methionine, choline and hexamethylenetetramine. The last three compounds were also examined in combination with ornithine.

The following bacteria were isolated and tested: four strains of *Bact. coli*, two strains of non lactose fermenting coliform bacteria, seven strains of *Streptococcus faecalis*, two strains of *Strep. mutis*, one strain of *Staphylococcus albus*, one strain of *Lactobacillus acidophilus*, fifteen unidentified strains of Gram positive cocci and diplococci, Gram negative oval cocci and Gram positive bacilli, some of them spore bearers.

To investigate whether the ability to form nicotinamide from ornithine depends on the intact structure of the bacterial cell, *Bact. coli* grown on agar slopes were washed off with saline and mechanically disintegrated by the method of Curran & Evans (1942). The supernatant fluid was removed in the centrifuge, the residue washed three times with saline, suspended in an ammonium lactate medium and used for incubation with ornithine. In some experiments 0.001 N and 0.0001 N NaCN was added. The effect of pH on nicotinamide formation from ornithine by disintegrated *Bact. coli* cells was studied by varying the pH of phosphate buffers added to the ammonium lactate medium from 7.4 to 5.5. To examine whether the nicotinamide compound synthesized by *Bact. coli* was nicotinic acid or nicotinamide, an assay was carried out with a strain of *Leuconostoc mesenteroides* (A.T.C.C. no. 9135) by the method of Johnson (1945), in addition to the method using *Lactobacillus arabinosus* which does not distinguish between these two compounds. The strain of *Leuconostoc mesenteroides*, kindly supplied by Dr B. C. Johnson of the University of Illinois, proved to be much more sensitive to nicotinic acid than to nicotinamide.

RESULTS

Experiments on rats

From Table 1 it is evident that tryptophan administration causes a rise of urinary nicotinamide methochloride elimination in albino and hooded rats. This rise is markedly greater after oral than after parenteral administration.

Table 1 *Average rise in urinary nicotinamide methochloride elimination after oral and intraperitoneal administration of DL tryptophan to two strains of rats*

(DL-Tryptophan (100 mg) administered to each rat NM=nicotinamide methochloride Figures in brackets give the range of values for individual rats)

Strain		Average daily NM elimination on 3 predosing days ($\mu\text{g/day}$) (Av of 6)	Rise in NM elimination after tryptophan administration (μg)			
			Oral*	Parenteral	Oral	Parenteral†
			(Av of 3)	(Av of 6)	(Av of 6)	(Av of 3)
Albino		186 (87-281)	197 (109-297)	77 (57-105)	206 (129-316)	76 (67-81)
Hooded		768 (579-1081)	346 (129-525)	121 (89-138)	323 (241-393)	124 (115-132)

* Beginning with oral administration

† Beginning with parenteral administration

Table 2 *Effect of succinylsulphathiazole on the increase of urinary nicotinamide methochloride elimination following oral administration of tryptophan*

(DL-Tryptophan (100 mg) administered to each rat NM=nicotinamide methochloride Figures in brackets give the range of values for individual rats)

Strain	No of animals	Before succinylsulphathiazole administration		During succinylsulphathiazole administration	
		Average daily NM output on 3 predosing days ($\mu\text{g/24 hr}$)	Increase of NM output on giving tryptophan (μg)	Average NM output on 3 predosing days ($\mu\text{g/24 hr}$)	Increase of NM output on giving tryptophan (μg)
Albino	3	209 (130-320)	253 (210-280)	41 (17-81)	66 (41-96)
Hooded	3	901 (710-1017)	508 (427-602)	144 (89-212)	87 (28-146)

The rise in urinary nicotinamide methochloride output caused by tryptophan (Table 2) is distinctly diminished when the basic nicotinamide methochloride elimination is reduced by feeding succinylsulphathiazole to albino or hooded rats. There is, however, one other experiment with one albino and one hooded rat in which the basic nicotinamide methochloride elimination is not markedly affected by succinylsulphathiazole. In this experiment the extra nicotinamide methochloride elimination caused by tryptophan was the same as before the succinylsulphathiazole administration. The administration of sulphathiazole as opposed to that of the succinyl compound did not markedly affect the increase in nicotinamide methochloride elimination. The concentration of total and free drug in the blood after administration of 1 g/day of succinylsulphathiazole was as follows (average of 3) total, 7.5 $\mu\text{g/ml}$, free, 5.8 $\mu\text{g/ml}$, after feeding of 0.1 g/day of sulphathiazole the respective figures were total, 52.0 $\mu\text{g/ml}$, free, 47.3 $\mu\text{g/ml}$.

When L-ornithine (0.2 g) or L-proline (0.2 g) was given to rats of both strains orally or parenterally no increase in urinary nicotinamide methochloride elimination was observed. The same negative result was obtained after administration by both routes of 4 aminovaleric acid (0.5 g) to rats of both strains.

Experiments on bacteria

Effect of tryptophan Addition of tryptophan to casein hydrolysate (Barton-Wright, 1944) or ammonium lactate media (Fildes, 1938) in concentrations of 0.004-0.0001 M markedly increased growth and acid production of two pure *Bact. coli* strains isolated from human faeces (Table 3). The maximum effect was observed in concentrations of 0.0006-0.0001 M. The production of nicotinamide/unit growth, however, was not affected. In pure cultures of *Strep. faecalis* (Table 4) growth and acid production were stimulated by tryptophan in concentrations of 0.002-0.004 M, and the disappearance of nicotinamide observed at lower concentrations came to an end at tryptophan concentrations > 0.001 M.

When various *Proteus* strains were incubated aerobically in the ammonium lactate medium in the presence of DL-tryptophan, pigments were formed varying in hue from light yellow to dark brown according to the type of organism (*Proteus morganii* 111, *Proteus vulgaris* 401, 3156, 5887) (all NCTC) and increasing in intensity with increasing concentrations of tryptophan. To examine these pigments *Proteus morganii* 111 was incubated aerobically at 37° in large flat bottles in ammonium

Table 3 *Effect of tryptophan on growth, acid production and nicotinamide formation by Bact coli 2 and 4 in Barton-Wright and ammonium lactate media*

Organism	Medium Tryptophan concentration (M)	Barton Wright				Ammonium lactate			
		Growth (arbitrary units)	Acid formed/ml (ml 0.1N NaOH)	Nicotinamide formed/unit acid		Growth (arbitrary units)	Acid formed/ml (ml 0.1N NaOH)	Nicotinamide formed/unit acid	
				($\mu\text{g/ml}$)	(% of control)			($\mu\text{g/ml}$)	(% of control)*
<i>Bact coli 2</i>	0	++	0.075	0.074	100	++	0.068	0.067	100
	10^{-4}	++	0.078	0.082	105	++	0.072	0.070	100
	2×10^{-4}	+++	0.077	0.080	104	+++	0.080	0.084	106
	4×10^{-4}	+++	0.083	0.082	99	+++	0.082	0.085	104
	5.8×10^{-4}	+++	0.084	0.080	95	+++	0.081	0.085	106
	10^{-3}	++++	0.094	0.095	100	++++	0.115	0.115	100
	2×10^{-3}	+++	0.084	0.080	95	+++	0.085	0.078	93
	2.7×10^{-3}	+++	0.073	0.075	102	++	0.070	0.064	93
	4×10^{-3}	++	0.072	0.070	97	++	0.065	0.060	94
<i>Bact coli 4</i>	0	+	0.033	0.050	100	+	0.030	0.045	100
	10^{-4}	+	0.040	0.055	91	+	0.036	0.050	91
	2×10^{-4}	+	0.042	0.060	95	+	0.044	0.060	90
	4×10^{-4}	++	0.045	0.064	94	++	0.044	0.062	93
	5.8×10^{-4}	++	0.058	0.078	90	++	0.055	0.080	97
	10^{-3}	++	0.045	0.065	96	++	0.042	0.059	93
	2×10^{-3}	+	0.040	0.060	100	+	0.036	0.050	91
	2.7×10^{-3}	+	0.036	0.050	92	+	0.035	0.048	90
	4×10^{-3}	+	0.030	0.045	100	+	0.030	0.041	90

* In Tables 3, 5, 6, 7, 9, 10, 11 and 12 the values for nicotinamide formed/unit growth (percentage of control) are obtained by dividing the values for nicotinamide formed/ml medium by the values for the acid produced/ml. The values thus obtained for a tested substance are divided by those obtained for the control and multiplied by 100.

Table 4 *Effect of tryptophan on growth, acid production and nicotinamide formation by Streptococcus faecalis in a casein hydrolysate medium containing nicotinic acid*

Concentration of tryptophan (M)	Growth (arbitrary units)	Acid production/ ml medium (ml 0.1N- NaOH)	Nicotin- amide formed ($\mu\text{g/ml}$)
0	+++	0.27	0
10^{-4}	++	0.24	~0.005
2×10^{-4}	++	0.25	~0.005
4×10^{-4}	++	0.25	~0.003
6×10^{-4}	++	0.25	~0.003
10^{-3}	+++	0.28	0
2×10^{-3}	++++	0.304	0
3.3×10^{-3}	++++	0.304	0
4×10^{-3}	++++	0.314	0

lactate containing 0.02M-DL-tryptophan for 48 hr. The medium, which had turned dark brown during incubation, was acidified with HCl to a final concentration of about 0.4% (w/v). Part of the pigment precipitated and was filtered off (A). The filtrate was extracted with ether until the extract was colourless. The ether was dried with Na_2SO_4 and evaporated, the residue forming an amorphous orange powder (B). The ether-extracted aqueous solution was then exhaustively extracted with *iso*-butanol. The extract was dried with Na_2SO_4 and the *iso*butanol was evaporated to a small volume

and kept at low temperature. An amorphous dark brown precipitate (C) formed which was possibly identical with A. Filter-paper chromatography of the aqueous medium after incubation revealed the presence of two distinct pigments, a yellow band with yellowish fluorescence and a brown band with pinkish fluorescence. Larger amounts of these pigments will have to be produced before more details can be given.

Effects of other compounds. When the synthesis of nicotinamide by *Bact coli* from amino acids other than tryptophan was tested (Table 5) ornithine was found by far the most efficient, whilst some increase in nicotinamide formation was shown by arginine and glutamine. None of these three amino acids markedly increased growth or acid production of the bacteria. None of the other amino acids affected nicotinamide formation to a marked degree. The optimal concentration of ornithine was between 6×10^{-4} and 1×10^{-3} M. The increase in nicotinamide formation per unit growth as compared with the control varied from two and a half to fifteen times.

Neither methionine nor choline (Table 6) affected the nicotinamide synthesis by *Bact coli*. When, however, these compounds were added to an ammonium lactate medium containing 10^{-3} M ornithine, they caused a marked rise of the nicotinamide formation as compared with that caused by ornithine alone.

Table 5 *Effect of different amino acids on growth, acid production and nicotinamide formation in ammonium lactate medium by Bact coli 3c and 4c*

Compound tested	Concentration of amino acid (M)	Bact coli 3c				Bact coli 4c			
		Growth (arbitrary units)	Acid produced/ml (ml 0.1N-NaOH)	Nicotinamide formed/unit acid		Growth (arbitrary units)	Acid produced/ml (ml 0.1N-NaOH)	Nicotinamide formed/unit acid	
				($\mu\text{g}/\text{ml}$)	(% of control)			($\mu\text{g}/\text{ml}$)	(% of control)
None	0	++	0.116	0.085	100	+++	0.147	0.125	100
L-Ornithine dihydrochloride	2×10^{-4}	++	0.119	0.150	173	+++	0.139	0.150	127
	2.5×10^{-4}	++	0.114	0.150	181	+++	0.139	0.175	148
	5×10^{-4}	++	0.121	0.175	197	+++	0.150	0.225	177
	10^{-3}	++	0.120	0.275	314	+++	0.150	0.325	254
	2×10^{-3}	++	0.121	0.240	271	+++	0.150	0.275	216
	4×10^{-3}	++	0.121	0.200	226	+++	0.150	0.225	177
L-Arginine nitrate	5×10^{-4}	++	0.121	0.150	170	+++	0.163	0.175	126
	6.6×10^{-4}	++	0.122	0.150	169	+++	0.164	0.180	128
L-Proline	5×10^{-4}	++	0.126	0.090	98	++++	0.171	0.150	101
	6.6×10^{-4}	++	0.121	0.090	101	++++	0.170	0.150	102
L-Leucine	5×10^{-4}	+++	0.141	0.090	88	++++	0.169	0.150	103
	6.6×10^{-4}	+++	0.146	0.100	93	++++	0.168	0.150	103
L-Isoleucine	5×10^{-4}	++++	0.152	0.100	90	++++	0.171	0.150	102
	6.6×10^{-4}	++++	0.157	0.100	88	++++	0.172	0.150	102
L-Valine	5×10^{-4}	+++	0.141	0.085	82	++++	0.167	0.125	88
	6.6×10^{-4}	+++	0.143	0.100	96	++++	0.177	0.125	84
None	0	++	0.103	0.163	100	++	0.117	0.140	100
L-Ornithine dihydrochloride	10^{-3}	++	0.100	0.595	342	++	0.110	0.520	400
DL-Glutamine	5×10^{-4}	++	0.113	0.255	172	++	0.125	0.210	141
	6.6×10^{-4}	++	0.110	0.270	154	++	0.130	0.230	148
DL-Glutamic acid	5×10^{-4}	+++	0.138	0.185	85	+++	0.150	0.145	82
	6.6×10^{-4}	+++	0.135	0.195	90	+++	0.145	0.135	76
L-Citrulline	5×10^{-4}	++	0.133	0.205	98	+++	0.153	0.130	71
	6.6×10^{-4}	++	0.130	0.205	99	+++	0.145	0.130	76
None	0	—	—	—	—	++	0.061	0.020	100
L-Ornithine dihydrochloride	10^{-3}	—	—	—	—	++	0.058	0.300	1542
4-Aminovaleric acid	5×10^{-4}	—	—	—	—	+++	0.061	0.020	100
	10^{-3}	—	—	—	—	+++	0.062	0.020	98
	2×10^{-3}	—	—	—	—	+++	0.059	0.020	102

Table 6 *Effect of choline and methionine alone and in combination with ornithine on growth, acid production and nicotinamide formation by Bact coli 4c in ammonium lactate medium*

Compound tested	Concentration of choline or methionine (M)	Growth (arbitrary units)	Acid produced/ml medium (ml 0.1N-NaOH)	Nicotinamide formed/unit acid	
				($\mu\text{g}/\text{ml}$)	(% of control)
None	0	+	0.056	0.010	100
L-Ornithine dihydrochloride (10^{-3} M)	0	+	0.052	0.038	390
	10^{-4}	+	0.055	0.010	102
	2×10^{-4}	+	0.057	0.010	98
	5×10^{-4}	+	0.056	0.010	100
L-Ornithine dihydrochloride (10^{-3} M) + choline chloride	10^{-3}	+	0.055	0.010	102
	10^{-4}	+	0.051	0.034	370
	2×10^{-4}	+	0.052	0.048	524
	5×10^{-4}	+	0.051	0.056	615
	10^{-3}	+	0.051	0.040	439
	10^{-4}	+	0.054	0.010	104
DL-Methionine hydrochloride	2×10^{-4}	+	0.056	0.010	100
	5×10^{-4}	+	0.057	0.010	98
	10^{-3}	+	0.057	0.010	98
	10^{-4}	+	0.054	0.032	334
	2×10^{-4}	+	0.050	0.036	404
L-Ornithine dihydrochloride (10^{-3} M) + DL-methionine hydrochloride	5×10^{-4}	+	0.053	0.058	613
	10^{-3}	+	0.052	0.048	519

Table 7 *Effect of hexamethylenetetramine on growth, acid production and nicotinamide formation by Bact coli 4c in ammonium lactate medium*

Compound tested	Concentration of hexamethylene tetramine (M)	Growth (arbitrary units)	Acid produced/ml medium (ml 0.1 N-NaOH)	Nicotinamide formed/unit acid	
				(μg /ml medium)	(% of control)
None	0	++	0.040	0.025	100
L Ornithine dihydrochloride (10^{-3} M)	0	++	0.048	0.20	695
Hexamethylenetetramine	10^{-5}	+	0.032	0.02	100
	3×10^{-5}	+	0.034	0.02	98
	10^{-4}	+	0.033	0.02	100
	3×10^{-4}	+	0.033	0.02	100
	10^{-3}	+	0.033	0.015	75
L Ornithine dihydrochloride (10^{-3} M) + hexamethylenetetramine	10^{-5}	+	0.050	0.185	617
	3×10^{-5}	+	0.052	0.185	617
	10^{-4}	+	0.052	0.175	564
	3×10^{-4}	+	0.050	0.170	565
	10^{-3}	+	0.050	0.170	565

Table 8 *Nicotinamide synthesis from ornithine by disintegrated cells of Bact coli 3c and 4c in saline and the effect of cyanide on this synthesis*

Concentration of L ornithine dihydrochloride (M)	Concentration of NaCN (M)	Strain	
		Bact coli 3c Nicotinamide content (μg /ml)	Bact coli 4c Nicotinamide content (μg /ml)
0	0	0.043	0.037
5×10^{-4}	0	0.060	0.042
10^{-3}	0	0.085	0.043
5×10^{-4}	10^{-3}	0.043	0.035
5×10^{-4}	10^{-4}	0.045	0.035

Hexamethylenetetramine (Table 7) in concentrations of 10^{-5} to 10^{-3} M slightly inhibited growth and acid production, and nicotinamide formation to a small degree at the highest concentrations tested.

Parallel assays of ammonium lactate media in which nicotinamide had been synthesized from ornithine by *Bact coli* were carried out by the method of Barton-Wright (1944) using *Lb arabinosus* and that of Johnson (1945) using *Leuconostoc mesenteroides*. While the former does not distinguish between nicotinic acid and nicotinamide, the strain used in the latter was much more sensitive to the acid than to the amide. In fifty two samples of these media in which the Barton Wright assay had revealed a combined nicotinamide and nicotinic acid content of 0.085–0.325 μg /ml, the Johnson assays gave values of less than 0.01 μg /ml, the lowest amount measurable by this strain. This shows that the compound synthesized was probably exclusively nicotinamide.

Experiments with disintegrated cells Table 8 shows that the formation of nicotinamide from ornithine probably does not depend on the intactness of the cell, but is completely inhibited by cyanide. In spite of the fact that the disintegration was done as thoroughly as possible it cannot be excluded that

some cells have survived and are responsible for the activity of the debris. Examinations with disintegrated cells in saline of pH 7.4–5.0 showed that nicotinamide formation from ornithine by cell debris varied very little in this range of pH.

Experiments with mixed cultures When mixed cultures from the contents of the rat's caecum were incubated in ammonium lactate medium with either ornithine or tryptophan (Table 9) there was in all experiments a marked rise of the synthesis of nicotinamide as compared with that of the control. The extent of the rise varied, but in each individual case the increase with ornithine was higher than that with tryptophan.

In casein hydrolysate medium (Table 10) the increase was less marked, and the difference between the effects of ornithine and tryptophan was smaller than in ammonium lactate medium.

The formation of nicotinamide from ornithine or tryptophan by the mixed cultures was almost the same under aerobic and anaerobic conditions (Table 11).

Drying of the mixed cultures (Table 12) affected to some extent the nicotinamide synthesis from tryptophan and ornithine. Whilst the ornithine effect was reduced by about 50%, the tryptophan effect was almost completely abolished.

Table 9 *Average acid formation and nicotinamide production by mixed cultures from the rat's caecum grown in ammonium lactate medium aerobically in the absence and presence of tryptophan and ornithine*

(Figures in brackets give the range of values for individual samples)

No of exp	Compound added	Acid formed/ml medium (ml 0.1N-NaOH)	Nicotinamide formed/unit acid	
			(μ g/ml medium)	(% of control)
7	None	0.080 (0.050-0.152)	0.020 (0.010-0.030)	100 (100)
	DL Tryptophan	0.080 (0.062-0.124)	0.038 (0.018-0.050)	195 (134-302)
	L Ornithine dihydrochloride	0.075 (0.053-0.120)	0.049 (0.030-0.070)	290 (157-472)

Table 10 *Influence of medium (ammonium lactate or casein hydrolysate) on the nicotinamide formation from tryptophan or ornithine by mixed cultures of bacteria from the rat's caecum grown aerobically*

(Figures in brackets give the range of values for individual samples)

Medium	No of exp	Compound added	Acid formed/ml medium (ml 0.1N NaOH)	Nicotinamide formed/unit acid	
				(μ g/ml medium)	(% of control)
Ammonium lactate	3	None	0.069 (0.050-0.105)	0.027 (0.025-0.030)	100 (100)
		DL Tryptophan	0.079 (0.062-0.110)	0.045 (0.040-0.050)	151 (134-166)
		L Ornithine dihydrochloride	0.072 (0.055-0.100)	0.057 (0.050-0.070)	211 (157-296)
Casein hydrolysate	3	None	0.087 (0.049-0.110)	0.058 (0.035-0.075)	100 (100)
		DL Tryptophan	0.091 (0.052-0.110)	0.080 (0.065-0.090)	139 (113-173)
		L Ornithine dihydrochloride	0.094 (0.052-0.116)	0.088 (0.075-0.095)	149 (120-158)

Table 11 *Influence of anaerobiosis on nicotinamide formation from tryptophan or ornithine by mixed cultures of bacteria from the rat's caecum in ammonium lactate medium*

(Figures in brackets give the range of values for individual samples)

Condition of incubation	No of exp	Compound added	Acid formed/ml medium (ml 0.1N NaOH)	Nicotinamide formed/unit acid	
				(μ g/ml medium)	(% of control)
Aerobic	3	None	0.086 (0.050-0.152)	0.027 (0.025-0.030)	100 (100)
		DL Tryptophan	0.084 (0.062-0.124)	0.047 (0.045-0.050)	171 (134-220)
		L Ornithine dihydrochloride	0.078 (0.055-0.120)	0.050 (0.050)	197 (157-255)
Anaerobic	3	None	0.059 (0.037-0.100)	0.020 (0.020-0.025)	100 (100)
		DL Tryptophan	0.054 (0.037-0.085)	0.040 (0.040)	165 (170-235)
		L Ornithine dihydrochloride	0.050 (0.028-0.086)	0.041 (0.035-0.047)	221 (205-230)

Non coliform bacteria isolated from the caeca of various rats were tested for the formation of nicotinamide from tryptophan either by incubation together with *Bact coli* or by addition of *Bact coli* to the medium in which the non coliform organisms had been incubated. Not one showed a positive effect.

DISCUSSION

The *in vivo* experiments on the rat showing a greater nicotinamide methochloride elimination after oral than after parenteral administration of tryptophan are in agreement with the findings of Schweigert & Pearson (1947) and favour the conception that the

Table 12 *Influence of drying on the nicotinamide formation from tryptophan or ornithine by mixed cultures of bacteria from the rat's caecum grown aerobically in ammonium lactate medium*

(Figures in brackets give the range of values for individual samples)

Condition of culture	No of exp	Compound added	Acid formed/ml medium	Nicotinamide formed/unit acid	
			(ml 0.1 N NaOH)	(μ g/ml medium)	(% of control)
Fresh	3	None	0.079 (0.062-0.105)	0.016 (0.010-0.025)	100 (100)
		DL Tryptophan	0.083 (0.069-0.110)	0.035 (0.032-0.040)	230 (153-302)
		L-Ornithine dihydrochloride	0.075 (0.053-0.100)	0.056 (0.040-0.070)	395 (296-472)
Dry	3	None	0.056 (0.031-0.078)	0.020 (0.014-0.026)	100 (100)
		DL-Tryptophan	0.053 (0.031-0.080)	0.024 (0.018-0.032)	120 (116-126)
		L-Ornithine dihydrochloride	0.051 (0.027-0.073)	0.045 (0.025-0.056)	249 (192-305)

intestinal flora is concerned with conversion of tryptophan into nicotinamide. But they do not exclude a participation of the rat tissue in this conversion, both processes may occur simultaneously. However, the participation of the intestinal flora is also favoured by the findings of Krehl *et al.* (1946) that the growth-restricting effect of tryptophan-deficient diets in growing rats can be reduced by feeding carbohydrates which cause a change in the intestinal flora. The inhibiting effect of succinylsulphathiazole on the increase of nicotinamide methochloride output caused by tryptophan equally favours the participation of the intestinal flora in the conversion of tryptophan into nicotinamide. The effect of succinylsulphathiazole on the rate of tryptophan-nicotinamide conversion was not always observed, it was not observed when the administration of the drug failed to reduce the basic nicotinamide methochloride elimination, thus confirming the connexion between tryptophan conversion and intestinal flora. As in human beings (Ellinger, Coulson & Benesch, 1944, Ellinger, Benesch & Kay, 1945) nicotinamide methochloride elimination was not markedly affected by sulphathiazole administration in the rat although the blood level of total and free sulphathiazole was much higher after sulphathiazole than after succinylsulphathiazole administration. Also the rise in nicotinamide methochloride elimination caused by tryptophan was reduced only by succinylsulphathiazole, and not by sulphathiazole. This indicates strongly that the drop after succinylsulphathiazole administration is not due to a process occurring in the tissue, and that the tryptophan nicotinamide conversion occurs at least to a large extent in the intestinal flora which is much more affected by succinylsulphathiazole than by sulphathiazole. The results of the experiments using succinylsulphathiazole are in apparent disagreement with

those reported by Spector (1948), who found after feeding 0.2 g of succinylsulphathiazole a greater conversion of tryptophan to nicotinic acid and greater recovery of added nicotinic acid. This increase cannot be explained, but the failure to find a reduced conversion is probably due to entirely inadequate doses of succinylsulphathiazole used in these experiments. The minimum daily dose of succinylsulphathiazole found to reduce markedly the *Bact. coli* population of the intestines in about two thirds of the experimental animals is in the region of 1 g (Ellinger, 1948). There is no direct evidence for any conversion of tryptophan into nicotinamide in the tissues of vertebrates with the exception of experiments by Schweigert, German & Garber (1948), who found in developing chick embryos a significantly increased nicotinic acid content after injection of tryptophan. On the whole the conversion of tryptophan into nicotinamide in the rat seems to be very incomplete, since the increase in urinary nicotinamide methochloride elimination caused by 100 mg tryptophan corresponds to about that caused by 1-2 mg of nicotinamide. Moreover, the conception that the intestinal flora is concerned with the tryptophan nicotinamide conversion is further supported by the *in vitro* experiments in which, without exception, the nicotinamide production by mixed cultures from the caecum was increased on addition of tryptophan to the medium.

The chemical mechanism of the tryptophan-nicotinamide conversion is obscure. Whilst of the known mammalian tryptophan metabolites kynurenic acid has been shown to be ineffective in increasing nicotinamide methochloride output in rats (Rosen *et al.* 1947), kynurenine which might be an intermediate in tryptophan metabolism in normal animals, and is an end product in pyridoxine deficient animals, seems to increase the nicotinic acid content of a

mutant strain of *Neurospora* (Beadle, *et al* 1947) The structure of kynurenine as *o*-aminophenacylglycine has been proved by Butenandt, Weidel & Neckel (1944) by synthesis If this structure for kynurenine is correct, its conversion either into a pyridine 3-carboxylic acid or into ornithine can hardly be understood It has been shown that 2-amino-3-hydroxybenzoic acid can replace tryptophan or nicotinamide in some mutant strains of *Neurospora* (Mitchell & Nyc, 1948, Bonner, 1948) The chemical mechanism of this effect is obscure, and a direct conversion of 2 amino-3-hydroxybenzoic acid into nicotinamide seems improbable

There is no evidence available so far that ornithine can be formed from tryptophan, but if tryptophan is directly converted into nicotinamide a reductive cleavage of the pyrrole ring of tryptophan in the almost anaerobic atmosphere of the lower intestines may cause the formation of ornithine from tryptophan

The possibility of such a cleavage, which is not supported by any known parallel reaction in biological systems, has still to be proved, and ornithine has to be isolated as an intermediate of such a conversion of tryptophan into nicotinamide

The experiments on *Bact coli*, wherein the formation of nicotinamide seems to be mainly responsible for the biosynthesis of nicotinamide in the intestines (Ellinger & Emmanuelowa, 1946), prove without any doubt that ornithine is the only one of the amino acids tested which facilitates the bacterial synthesis of nicotinamide to a considerable degree The only other amino acids which show a similar but much smaller effect, namely arginine and glutamine, are probably converted to ornithine, the former by an enzymic action similar to that described by Hira (1936), the latter by a reductive process

The failure of the rat to convert ornithine into nicotinamide *in vivo* is not in conflict with the conception that it is concerned in the biosynthesis of nicotinamide Ornithine is so unstable in the mammalian body that its natural occurrence in mammals has not been recognized until its importance for the formation of urea was demonstrated by Krebs & Henseleit (1932a, b, 1934) It seems to be quickly used up in the formation of arginine It might be expected that one of the intermediates postulated in its conversion into nicotinamide may be effective in this respect Klein & Linser (1932), who found ornithine and proline to be active as precursors of trigonelline in *Trigonella foenum-graecum*, suggested the intermediate formation of 1 hydroxy-4 aminovaleric acid, 4 aminovaleric acid, glycine and nicotinic acid, and Guggenheim (1940) omitting the 1 hydroxy-4 aminovaleric acid, but including 4 aminobut-1-ene 1 carboxylic acid between 4 aminovaleric acid and glycine, suggested a similar

pathway The failure of 4 aminovaleric acid to be converted into nicotinamide *in vivo* in the rat and *in vitro* by *Bact coli* shows that this compound cannot be an intermediate of the conversion This mechanism needs further investigation The results with bacteria differ from those of Klein & Linser (1932, 1933a, b) with *Trigonella* in two respects proline or glutamic acid are inactive in bacteria and hexamethylenetetramine does not increase the nicotinamide production That a methylating process is involved in the ornithine-nicotinamide conversion is suggested by the increase in nicotinamide formation when methyl donors such as choline or methionine are available

The fact that *Bact coli* forms indole (Herzfeld & Klinger, 1915) under aerobic and 3-indolepropionic acid under anaerobic conditions (Woods, 1935), but fails in pure culture to convert tryptophan into nicotinamide, suggests that *Bact coli* cannot split the tryptophan molecule into ornithine This cleavage might be performed by other intestinal bacteria as shown by the results obtained with mixed cultures from rat caecum It was, however, not possible to isolate these organisms which do not appear to stand drying It is remarkable that the compound formed by the intestinal bacteria is nicotinamide and not the acid

The experiments reported strongly favour the conception that in the conversion of tryptophan into nicotinamide in the rat the intestinal bacteria are responsible to a considerable extent, but they do not exclude a participation of the rat's tissues in this process

SUMMARY

1 Administration of tryptophan causes an increase of urinary nicotinamide methochloride elimination in adult rats kept on a mixed diet This increase is markedly greater after oral than after parenteral administration

2 The increase in urinary nicotinamide methochloride output caused by tryptophan administration is markedly reduced in rats in which feeding of succinylsulphathiazole has produced a reduction in basal nicotinamide methochloride elimination, but not by sulphathiazole

3 Ornithine, proline and δ aminovaleric acid do not cause an increase of nicotinamide methochloride output in the rat

4 Ornithine, and to a lesser extent arginine and glutamine, increase the formation of nicotinamide by *Bact coli* Methionine and choline, which are inactive by themselves, increase the nicotinamide formation from ornithine by *Bact coli*, whilst hexamethylenetetramine is ineffective in this respect

5 The conversion of ornithine into nicotinamide by *Bact coli* seems not to depend on maintaining intact the structure of the bacterial cell

6 *Bact coli* in pure culture does not convert tryptophan into nicotinamide, but mixed cultures from the content of the rat's caecum perform this conversion

7 The compound formed by bacterial synthesis is nicotinamide and not nicotinic acid

8 The conversion of tryptophan into nicotinamide in the rat is discussed. It is suggested that the intestinal flora plays an important role in this process and that ornithine is an intermediate in this conversion

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The Measurement of Glucuronide Synthesis by Tissue Preparations

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To measure the ability of surviving tissue slices to form glucuronides of compounds such as menthol and phenol, Lipschitz & Bueding (1939) determined ether soluble compounds giving the Tollens colour reaction for glucuronic acid. Whilst this procedure yielded valuable information in their hands, it is inconvenient for routine purposes, and the lack of specificity in the colour reaction can be a serious disadvantage. It must also be borne in mind that not all glucuronides are as ether soluble as that derived from menthol. De Meio & Arnolt (1944), in their study of phenol conjugation by tissue slices, assumed the figure for combined phenol given by the method of Theis & Benedict (1924) to represent phenylsulphuric acid and phenylglucuronide. As shown below, it is doubtful whether the results of De Meio & Arnolt (1944) have any bearing on the problem of glucuronide synthesis.

It was considered that the study of the biological mechanism of glucuronide formation required a sensitive reaction, specific for a glucuronide in the presence of a large excess of the hydroxy compound. Following a suggestion made by Dr R T Williams (private communication), the possibility was investigated of determining *o*-aminophenyl- β -D-glucuronide (Williams, 1943) in the presence of free *o*-aminophenol by the reaction described by Bratton & Marshall (1939) for the estimation of sulphonamides. If the reaction was carried out according to their directions, in strongly acid solutions, the bluish pink given by the glucuronide was slow to develop, and when it had reached its full intensity an appreciable colour of similar shade was seen in parallel experiments with the free phenol. By careful control of pH it was found possible, not only to shorten the period for complete colour development with the glucuronide, but to eliminate interference by free *o*-aminophenol. To avoid having to adjust the pH of the solution after removal of proteins, methods of protein precipitation at the pH chosen for colour development were studied. Application of the complete procedure to the measurement of glucuronide synthesis by mouse liver slices is described below.

EXPERIMENTAL

The colour reaction

Diazotization and coupling with naphthylethylenediamine were carried out as described by Bratton & Marshall (1939). To 2 ml of the test solution were added 1 ml acid or buffer, followed at suitable intervals by 1 ml of each of the

following 0.1% NaNO₂, 0.5% ammonium sulphamate and 0.1% naphthylethylenediamine dihydrochloride. In later experiments, the concentration of NaNO₂ was reduced to half. When 1 ml of N-HCl or N-trichloroacetic acid was added to the reaction mixture, quantities of the order of 100 μ g *o*-aminophenol present as the glucuronide could readily be distinguished from larger amounts of the free phenol by the pink colour seen immediately after adding the coupling reagent. It was possible to detect much smaller amounts of the glucuronide if colour development was allowed to proceed to completion, the process being accelerated by warming, but under such conditions 100 μ g free *o*-aminophenol gave a strong pink colour. Table 1 shows the

Table 1 *Extinction coefficients, using different filters, of the azo colours produced by o-aminophenylglucuronide and by o-aminophenol*

(Colour development for 18 hr at 37° in presence of HCl 12 μ g *o*-aminophenylglucuronide (calc as free phenol) and 120 μ g *o*-aminophenol. Extinction coefficients measured with Hilger Spekker absorptiometer.)

Hilford filter no	Extinction coefficients	
	<i>o</i> -Aminophenyl glucuronide	<i>o</i> -Aminophenol
601	0.024	0.053
602	0.075	0.077
603	0.168	0.120
604	0.312	0.202
605	0.432	0.312
606	0.337	0.322
607	0.160	0.232
608	0.012	0.048

extinction coefficients with different filters in the Hilger Spekker absorptiometer after 18 hr colour development in HCl at 37°, and it can be seen that no filter permitted distinction between the two compounds. Hilford filter no 605 (yellow green) was selected for further work.

Freshly prepared aqueous solutions of pure *o*-aminophenol were colourless, but on standing there was rapid formation of an ether soluble brown pigment giving appreciable readings in the Spekker absorptiometer. Shaking the solution in an atmosphere of O₂ hastened pigment formation. Interference from this source was greatly reduced or entirely prevented by adding ascorbic acid to the solution. Ascorbic acid itself had no effect on the colour reaction for the glucuronide. The initial reaction of *o*-aminophenol with HNO₂ gives a pale yellow solution. This colour does not register on the Spekker absorptiometer with Hilford filter no 605.

The effects of pH and temperature on the colour development

Fig 1 shows the effect of varying the pH on the comparative intensities of the colours given by *o*-aminophenol

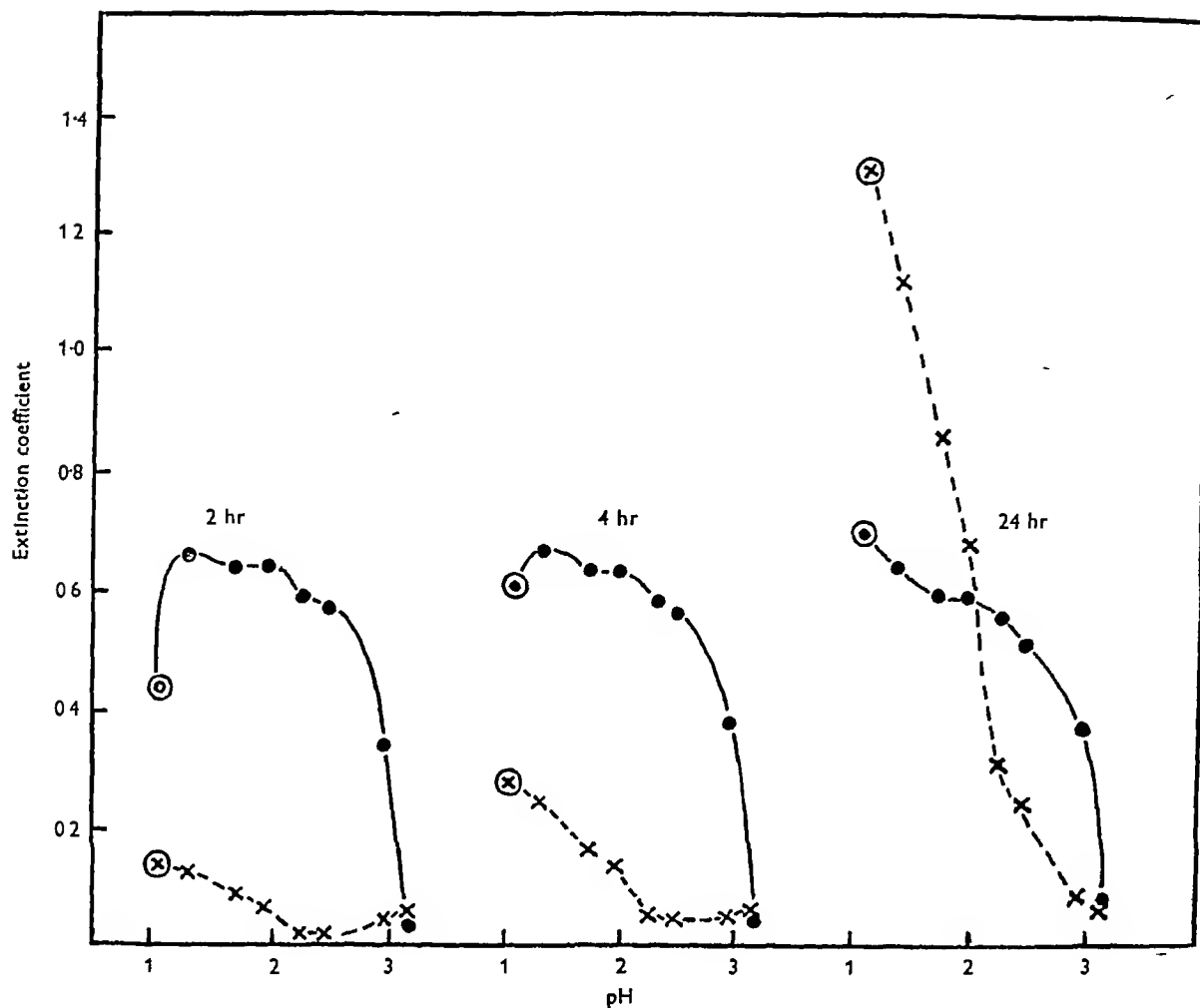


Fig 1 The effect of pH on colour development after different periods at 25° ●—●, 12 μ g *o* aminophenol as glucuronide, x—x, 120 μ g free *o* aminophenol (no ascorbic acid present) Encircled points, pH adjusted with HCl, all others, phosphate buffer

and its glucuronide after different periods at 25°. Except for experiments at the lowest pH, in which N HCl was used, the pH was adjusted by addition of approximately M phosphate buffer. As elsewhere in this paper, the amount of glucuronide is expressed in terms of the *o* aminophenol content. Above pH 2.25 no azo dye formation was seen with the free phenol after 4 hr incubation, the Spekker absorptiometer readings being accounted for entirely by the pigmented oxidation product. Above pH 2.50 the intensity of the colour given by the glucuronide fell sharply. On the basis of these results it seemed that pH 2.25–2.50 was the most favourable region for determining *o* aminophenyl glucuronide in the presence of excess of the free phenol. The use of agents other than phosphate for pH adjustment led to a similar conclusion. From Fig 2 it can be seen that within the desired pH region colour development was complete with the glucuronide in 2 hr at 25°. In the presence of HCl, on the other hand, the colour had not reached a maximum after 6 hr incubation. Azo dye formation by the free phenol below pH 2.25 can be clearly distinguished from oxidation alone at higher pH values in Fig 2.

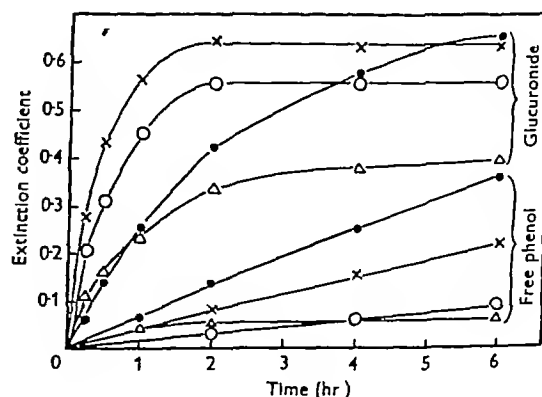


Fig 2 The effect of pH on the rate of colour development at 25° ●—●, pH 1.05 (HCl), x—x, pH 1.68 (phosphate buffer), ○—○, pH 2.25 (phosphate buffer), △—△, pH 2.94 (phosphate buffer) 12 μ g *o* aminophenol as glucuronide or 120 μ g free *o* aminophenol (no ascorbic acid present)

Fig 3 illustrates the effect of temperature on the colour development at pH 2.25. In general, increasing the temperature caused the maximum to be reached more quickly, but decreased the ultimate value, and fading occurred on prolonged incubation at high temperatures. Raising the temperature also increased the rate of oxidation of the free phenol in absence of ascorbic acid. At 25°, coupling of the diazotized glucuronide at pH 2.25 was as rapid as at 37°, and the former temperature was adopted for further work.

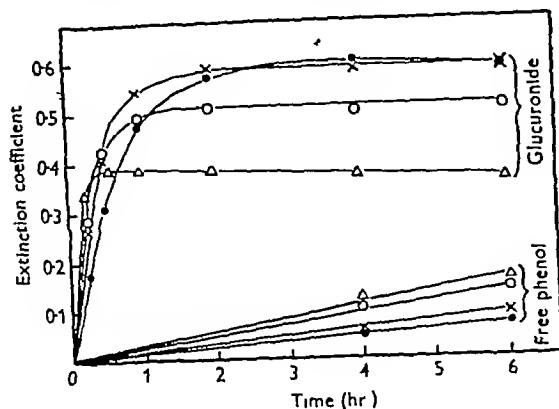


Fig 3 The effect of temperature on colour development at pH 2.25. ●—●, 16°, ×—×, 25°, ○—○, 37°, △—△, 49°. 12 µg *o* aminophenol as glucuronide or 120 µg free *o* aminophenol (no ascorbic acid present)

The absorption curve for the glucuronide under the selected conditions of colour development (2 hr at 25° and pH 2.25–2.50) was very similar to that shown in Table 1, and a straight-line relationship was observed between the absorptometer readings with Ilford filter no 605 and amounts of the glucuronide equivalent to 2–24 µg *o* aminophenol.

Protein precipitation

Solutions of sodium tungstate or trichloroacetic acid brought to pH 2.25 did not precipitate proteins until further additions of acid were made. The presence of phosphate buffer at the same pH did away with the necessity of making the precipitant more acid. Tungstic acid precipitated naphthylethylenediamine, but mixtures of trichloroacetic acid and phosphate buffer proved satisfactory in giving protein free solutions in which the colour reaction could be carried out directly. Trichloroacetic acid was without effect on the colour reaction under these conditions, in spite of the fact that used alone for colour development at pH 1 it gave variable results for a known amount of glucuronide.

The effect of varying the *o* aminophenol concentration on synthesis of the glucuronide by mouse liver slices

The slices (about 10 mg dry wt), suspended in bicarbonate Ringer solution (Krebs & Henseleit, 1932), were shaken in an atmosphere of 5% CO₂ in O₂ at 37°. The solution contained 0.02M lactate (Lipschitz & Bueding, 1939), 0.001M ascorbic acid and varying concentrations of *o* aminophenol, but no sulphate, MgSO₄ being replaced by an equivalent concentration of MgCl₂. Glucuronide synthesis was determined at varying periods for each concentration of *o* aminophenol, slices from a single animal being used for

each complete experiment. The course of the synthesis in representative experiments is shown in Fig 4, results being expressed as mg *o* aminophenol conjugated by 1 g dry weight of slices. Each point is the mean of three or four individual results.

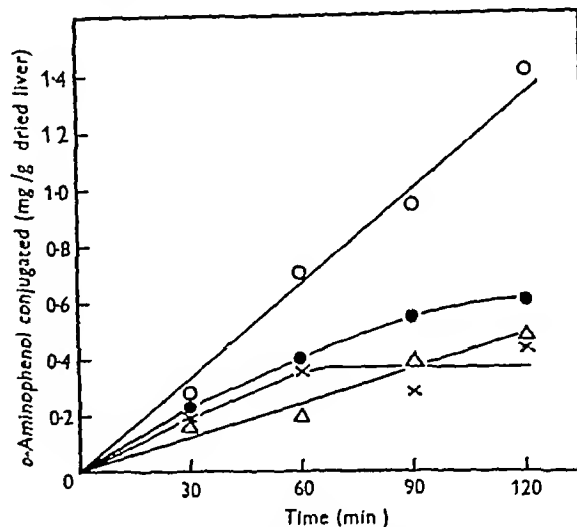


Fig 4 The course of glucuronide synthesis by mouse liver slices in various concentrations of *o* aminophenol. △—△, 0.005%, ○—○, 0.0025%, ●—●, 0.00125%, ×—×, 0.0005%

Variations in the initial slope may in part reflect individual variation in the ability of the liver to synthesize the glucuronide, but this factor can hardly explain all the differences in the shapes of the curves shown in Fig 4. It appears that the rate of synthesis remained constant for 2 hr in 0.0025% *o* aminophenol. Reducing the concentration caused the rate of reaction to fall off after incubation for 1 hr. Whilst in 0.0035% *o* aminophenol (results not shown) the reaction followed the same course as in 0.0025%, increasing the concentration to 0.005% caused an apparent decrease in the reaction rate. This is in agreement with the results of Lipschitz & Bueding (1939), who observed inhibition of glucuronide formation by excess alcohol or phenol.

Assay procedure for mouse liver slices

Into 25 ml conical flasks fitted with rubber stoppers were measured 2 ml bicarbonate Ringer, containing 0.02M lactate, 0.001M ascorbic acid and 0.0025% *o* aminophenol, but no sulphate. Ascorbic acid and the phenol were added as the solids, in that order. The latter was purified by sublimation before use. After passing a brisk stream of 5% CO₂ in O₂ into the flasks for 1 min they were stoppered and placed in a thermostatically controlled water bath at 37°. The contents were brought to atmospheric pressure by momentary release of the stoppers. Slices corresponding to approximately 10 mg dry weight were dropped into each flask which was again gassed for 2 or 3 sec before replacing the stopper. Shaking was commenced and continued for 1 hr. The slices were then removed for determination of the weight after drying at 110°. To the flask, 2 ml of a solution containing sodium trichloroacetate and phosphate were immediately added. This solution was prepared daily by mixing equal volumes of stock M trichloroacetate and M-phosphate, both adjusted to pH 2.25. The contents of the

flask were thoroughly mixed and poured into a 10 ml centrifuge tube. After centrifuging, 3 ml of the supernatant fluid were transferred to another tube, and to it was added 1 ml 0.05% NaNO_2 , followed after 3 min by 1 ml 0.5% ammonium sulphamate and, after another 2 min, by 1 ml 0.1% naphthylethylenediamine dihydrochloride. The tubes were placed in a bath at 25° for 2 hr and the intensities of the colours read with a Spekker absorptiometer against the reagents, using Ilford filter no. 605. Liver slices from normal mice gave zero readings after incubation in absence of *o* aminophenol, as did the phenol itself in the concentration used above when ascorbic acid was present. Variation in a single liver in synthetic activity from slice to slice, however, made it advisable to do determinations in quadruplicate (see below).

Errors in the assay

To test the precipitation procedure, a large number of mouse liver slices were shaken in bicarbonate Ringer solution of the composition described above except in that *o* aminophenol was omitted. After 1 hr the slices were removed and to 1 ml samples of the residual liquid was added 1 ml bicarbonate Ringer solution containing *o* aminophenylglucuronide equivalent to 9.2 μg of the free phenol. The removal of proteins and the colour reaction were carried out as described. The mean recovery in twenty two observations was 100%, and the standard deviation of a single observation from the mean 7%. This figure takes no account of the variable error arising from removal of slices in an actual assay. An overall measure of the variable error in the assay procedure, including that arising from variation from slice to slice in the synthetic activity of liver, was obtained from the individual results in a series of eight determinations, each done in quadruplicate, of the normal activity of adult mouse liver. The mean activity was 0.37 mg *o* aminophenol conjugated/g dry wt/hr, and the standard deviation of a single observation from the mean 17%.

The identity of the compound giving the colour reaction

It was borne in mind that part of the *o* aminophenol might be conjugated with sulphuric acid during the incubation with liver slices, and that the conjugate thus formed might give the colour reaction for *o* aminophenylglucuronide. *o* Aminophenylsulphuric acid was not available for study, but indirect evidence suggested that, if formed during the incubation, it did not interfere in the final determination.

In experiments in which mouse liver slices were incubated with *o* aminophenol in Ringer solution containing MgSO_4 , the final readings were, if anything, slightly lower than those obtained with slices from the same animal in sulphate free Ringer. This suggests that sulphate ion is not involved in the formation of the compound giving the colour reaction.

A comparative study of the stabilities of phenylglucuronide and phenylsulphuric acid to acid, using the colour reaction of Fohn & Ciocalteu (see Kerr, Graham & Levy, 1948), showed the former to be unchanged after 10 min at 100° in *N*-HCl, while the latter was completely hydrolyzed under these conditions. In phosphate buffer, pH 2.25, the degree of hydrolysis of phenylsulphuric acid was 50% after 10 min at 100°, and 25% after 2 hr at 25°. *o* Ammo-

phenylglucuronide, studied by the colour reaction described above, resembled phenylglucuronide in its stability to acid. The molar concentrations in these experiments were of the same order as those dealt with elsewhere in this paper, and excess acid was neutralized before proceeding with the appropriate colour reaction. The compound giving the colour reaction after incubation of *o* aminophenol with mouse liver slices was just as stable to acid as the authentic glucuronide. In these experiments, the incubation was done on a larger scale than usual and protein was removed with trichloroacetic acid. Samples were heated with HCl for varying periods, and excess acid neutralized before adding the buffer for colour development. It is considered unlikely that *o* aminophenylsulphuric acid would escape hydrolysis in such experiments. This is confirmed by the work of Burkhardt & Wood (1929), who found that although *o* aminophenylsulphuric acid was 'rather more difficult' to hydrolyze than phenylsulphuric acid, hydrolysis was detectable after boiling for a few seconds in dilute mineral acid. They also showed that diazotization in *N* HCl led to loss of the sulphate group.

DISCUSSION

De Meio & Arnolt (1944) observed tissue slices from one of their strains of rats to conjugate phenol in sulphate-free Ringer solution. Addition of sulphate, however, increased conjugation by liver, and the other strain of rats resembled guinea pigs (Bernheim & Bernheim, 1943) in that this ion was obligatory for phenol conjugation as determined by their method of assay. In the method of Theis & Benedict (1924) combined phenol is taken to be the difference between readings obtained before and after hydrolysis in 0.25 *N*-HCl for 10 min at 100°. If, as appears to be the case, De Meio & Arnolt (1944) carried out their hydrolysis in this way, their results did not include phenol conjugated with glucuronic acid, since pure phenylglucuronide was unchanged after 10 min at 100° in *N* HCl. Unless some other acid-labile phenol derivative was present, the results of De Meio & Arnolt must be considered to apply to formation of the sulphate only. In this case it would appear that in one of their strains of rats the tissue slices either contained or produced sufficient sulphate ion for a limited degree of conjugation. In our own procedure, synthesis of the compound giving the colour reaction for *o* aminophenylglucuronide was not stimulated by addition of sulphate, and this ion was omitted as a matter of routine.

SUMMARY

1 Small amounts of *o*-aminophenylglucuronide can be determined colorimetrically after diazotizing and coupling with naphthylethylenediamine. Interference with the colour reaction by comparatively large amounts of *o* aminophenol can be excluded if the colour is developed at pH 2.25, and

oxidation of *o* aminophenol is avoided when ascorbic acid is present. Trichloroacetic acid solutions brought to pH 2.25 precipitate proteins if phosphate buffer at the same pH is also present, permitting colour development without further pH adjustment.

2. The colour reaction has been applied to the measurement of glucuronide synthesis by mouse

liver slices. Rates of synthesis of the order of 0.7 mg *o* aminophenol conjugated/g dry weight of liver/hr were obtained at the optimum concentration of 0.0025 %.

The authors wish to express their gratitude to Miss Lynda M. H. Kerr, who carried out determinations of the stabilities of phenylglucuronide and phenylsulphuric acid, and to D. Love for technical assistance.

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The Frequency Distribution of the Zinc Concentrations in the Dental Tissues of the Normal Population

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In earlier work the author demonstrated the presence of zinc in the dental tissues (Cruickshank, 1936) and studied the increases occurring in persons suffering from active tuberculosis (Cruickshank, 1940). The present paper deals with the way in which zinc concentrations of dental tissues vary throughout the 'normal' population. The experimental results indicate that the population sampled was heterogeneous and divisible into two (or three) groups, each with its characteristic zinc concentrations. The significance of these findings is briefly discussed.

EXPERIMENTAL

Population and material sampled. Over a period of years, the author collected 'sound' teeth extracted, for orthodontic reasons, from secondary school children attending the Public Dental Clinic at Cambridge. All specimens were carefully examined and only fully calcified pre molars free from visible caries or defects were selected for analysis.

The children, boys and girls, ranged in age from 10 to 17 years, 80 % falling between 12 and 15 years. They can be regarded as a random sample of the pre adolescent secondary school population biased only in the sense that all had a degree of 'overcrowded' dentition calling for orthodontic extraction.

Zinc determinations. These were made by a modification of the dithizone extraction, ferricyanide iodine titration technique of Sylvester & Hughes (1936) reduced to a micro scale in order to permit quadruplicate or quintuplicate

analyses on the dentine and on the enamel of each single tooth specimen (Cruickshank, 1948). The standard deviation of the 250 standards (20 µg) run during the tests was ± 3.8 %. Interbatch variations were eliminated by 'batch scattering' the multiple analysis for each specimen. The total number of zinc estimations was about 1250.

Preparation of specimens. **Dentine.** The 'root' of each pre molar was cut off just below the crown with a fissure bur, dried to constant weight (105°), ashed in an electric furnace overnight (500°), cooled and pulverized. The four to five analyses were run on samples of this pulverized ash.

Enamel. The 'crown' was first heated in the electric furnace for 0.5 hr at 270° (pyrometer controlled) to char the dentine. When cool the 'neck' was pressed lightly into a surface of plasticine, surrounded with a 1 in. length of 1 in. diameter rubber tubing, and the hole filled with plaster of Paris. When set, the plaster containing and supporting the 'crown' was removed and trimmed to expose the 'neck'. The charred dentine was then cut out with a rose head bur. The remaining enamel 'cap' was freed by making a cruciform saw cut into the block and splitting apart, it was washed and ashed overnight (500°), and four to five samples of the pulverized ash were analyzed.

Examination of variables. It was intended to obtain experimental data for 100 persons but exigencies of the war reduced this to 70. The available data were first studied in relation to the known variables of (i) sex, (ii) age of the subject, (iii) position, and (iv) condition of the specimen. As already mentioned all specimens were 'pre molars' (the zinc contents of incisors, canines, pre molars and molars differ to some extent). The 'defectives' were sound teeth with no caries but with enamel of slightly less perfect

structure than average In view of the object of the experiment some knowledge of the effects of these accidental variables on zinc concentrations was imperative

RESULTS

The results are summarized in Table 1 There is some suggestion of systematic effects, for example in dentine the zinc concentrations in males are 5 % greater than in females and in enamel the zinc

by the enamel and dentine frequency curves it is clear that these 'zones' correspond to the *M*, *T* and *C* peaks already noted (Fig 1) These results are only suggestive, definite proof of the existence of such subgroups must await more extensive and elaborate surveys

Embryological factors Dentine and enamel arise from two distinct embryological layers, viz meso-derm and ectoderm, their calcification is effected by two independent groups of cells, the odonto-

Table 1 Summary of zinc concentrations in the teeth of the normal population classified in relation to certain known variables

Type of tooth	Dentine				Enamel			
	Males		Females		Males		Females	
	Mean Zn conc (mg /kg)		Mean Zn conc (mg /kg)		Mean Zn conc (mg /kg)		Mean Zn conc (mg /kg)	
	No	No	No	No	No	No	No	No
All	196	33	205	37	195	53	199	46
Normal	194	26	205	27	195	40	194	36
Defective	201	7	209	9	195	13	206	10
Uppers	198	17	208	20	195	22	188	23
Lower	194	14	204	16	190	17	208	17
Age of subject (yr)								
10 and 11	179	3	186	8	177	6	190	12
12 and 13	196	12	213	26	192	24	200	31
14 and 15	193	14	189	2	206	16	191	2
16 and 17	216	4	—	—	204	6	—	—

The s.d.'s of all means lie between ±20 and 30 mg /kg

concentrations of lower pre-molars are 15 % greater than for upper pre-molars In both dentine and enamel zinc concentrations appear to increase with age, rising respectively 12 and 15 % during a period of 6 to 7 years, but in all these averages there is a wide scatter and a large degree of overlapping The average zinc concentrations found in these experiments were some 20 % higher than those found previously in a mixed adult population (dentine 178, enamel 152 mg /kg) (Cruickshank, 1936)

Frequency distributions The observed zinc concentrations were ranged in order of ascending magnitude and the curves (Fig 1) plotted from a 3 point moving average across 5 mg /kg class intervals That for enamel gives a peak at 200 mg /kg (*M*) with subsidiaries at 170 mg /kg (*C*) and 220 mg /kg (*T*) That for dentine is apparently bimodal with a major peak at 190 mg /kg and another well developed peak around 220 mg /kg (*CT*)

A scatter diagram of the independently determined dentine and enamel zinc concentrations was next prepared and the frequency contours plotted (using a moving average across the 5 × 5 mg /kg two dimensional cells) There are three 'zones' of high frequency, two juxtaposed and the third discrete From the elevation of this 'surface' as given

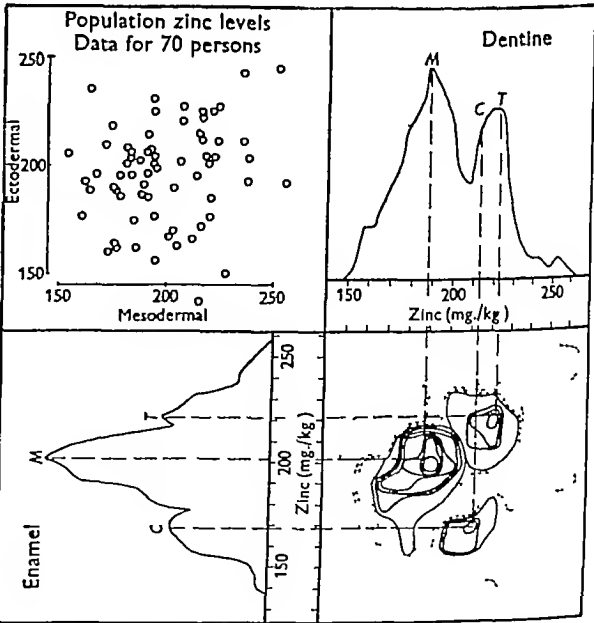


Fig 1 Frequency curves of the zinc concentrations in dentine and enamel, and frequency contours of the dentine enamel correlation surface

blasts and the ameloblasts There is therefore no *a priori* reason to anticipate a 'normal correlation' between the respective zinc levels of these two

calcified tissues Because of this the 'abnormalities' of the frequency surface and curves may have biological significance A suggested interpretation is that the population sampled was heterogeneous when classified in terms of the zinc concentrations of the two dental tissues examined These irregularities could not be accounted for in terms of the known variables described above

DISCUSSION

The main interest of the investigation lies in the demonstration of the possible existence of these population subgroups, other related work has

persons suffering from tuberculosis, in which condition there is evidence of increases in tooth zinc concentrations several years before any clinical manifestations of the disease appear (Cruickshank, 1940) Group *T* may therefore represent pre-tuberculosis subjects or alternatively persons prone to that disease

If, as seems possible, zinc plays some essential role in calcification processes, its variations in tuberculous and pre-tuberculous(?) persons would be understandable, for 'calcification' of the lesion is one form of resistance against the disease The therapeutic effects of zinc in tuberculosis are discussed by Ballard (1943)

It is of interest to compare the results of the present experiments with those of an independent investigation into serum alkaline phosphatase levels in normal and tuberculous persons (Murnaghan, 1946) Utilizing Murnaghan's data, Fig 2 was prepared to show the striking resemblance which exists between the tooth zinc and serum phosphatase distributions (note particularly the bimodal frequency curves) There is a further resemblance in that the zinc and phosphatase increases (in tuberculosis) are unaccompanied by any increase in serum calcium and phosphorus (Table 2) Such close parallelism can scarcely be accidental

Subgroup *C* is not discussed in this paper as there is as yet no experimental evidence of its nature or origin

SUMMARY

1 The zinc concentrations in the dentine and enamel of pre molars of a group of seventy secondary school children were determined by a modified dithizone ferricyanide-iodine titration method

2 After eliminating secondary variables there remained an apparent irregularity in the frequency distribution indicating possible division of the subjects into one major (*M*) and two minor (*T* and *C*) groups each in respect of their zinc concentrations One group (*T*) showed characteristics identical with those found in tuberculous subjects, characteristics which are known to appear years before clinical manifestation of the disease, it is therefore

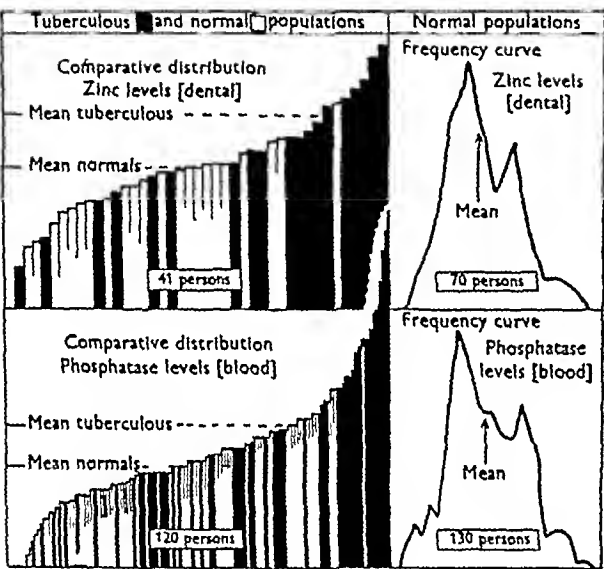


Fig 2 Resemblance between the distributions of the tooth zinc (0.5 x (dentine + enamel values)) and serum alkaline phosphatase levels in the normal population and in persons suffering from active tuberculosis (see also Table 2)

already indicated this possibility (Cherry, 1924, 1925, Cruickshank, 1939) If group *M* be taken as the mean type then group *T* differs from it in having a higher zinc concentration in both enamel and dentine This is exactly the difference found in

Table 2 The relative specificity of the dentine zinc and serum alkaline phosphatase increases in tuberculosis

	Normal		Tuberculous		Significant increase
Serum calcium (mg /100 ml)	11 03±0 547	(130)*	10 36±0 5	(55)*	No
Serum inorganic phosphorus (mg /100 ml)	3 38±0 509	(130)*	3 38±0 49	(55)*	No
Alkaline phosphatase (units/100 ml)	6 49±2 60	(130)*	9 05±4 4	(55)*	Yes (P= < 0 00006)
Dentine ash (g /100 g)	73 85±1 41	(41)	73 81±2 11	(74)	No
Dentine zinc (mg /kg)	178±21	(55)	192±26	(75)	Yes (P=0 0007)

All the results are given as mean ± s.d. and (in brackets) no. of specimens analyzed
* After Murnaghan (1946)

suggested that group *T* may represent pre-tuberculous or tuberculosis-prone persons

3 The close correspondence which exists between the tooth zinc and the serum alkaline phosphatase levels, both in health and in tuberculosis is discussed, and it is suggested that the role of zinc

in tuberculosis may be connected with calcification of the lesions

I wish to express my great indebtedness to Mr W Baird Grandison and his staff at Cambridge for very full and cordial co operation, and also to Mr Sampson for his technical skill in preparing the enamel specimens

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A Biochemical Study of *Pseudomonas prunicola* Wormald

1 PECTIN ESTERASE

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(Received 13 August 1948)

Certain species of *Pseudomonas* are responsible for the production of canker in stone fruit trees (Wormald, 1930) Erikson (1945), working with a strain of *Ps mors prunorum* Wormald, showed that the pathogen penetrated the tissues of the host intercellularly, plasmolysis of the cell contents and disintegration of the cell walls being followed by gradual invasion by the bacteria. This suggests that destruction of the pectic substances of the middle lamella of the host cells by extracellular pectic enzymes produced by the bacteria may play a primary part in facilitating the invasion of the tissues. Consequently, an investigation of the pectic enzymes of organisms involved in this invasion has been undertaken. The present paper describes investigations of an extracellular pectin esterase secreted by *Ps prunicola* Wormald, an organism causing a similar disease in cherry and pear trees.

EXPERIMENTAL

Organism For this work a strain of *Ps prunicola*, isolated from an infected pear tree at East Malling Research Station, was used. This is a Gram negative, non sporing rod, falling into Dowson's Group II (Dowson 1939), the type species of which is *Ps fluorescens* Migula.

Cultivation The organism was grown in Roux bottles on a medium consisting of a tryptic digest of casein containing 0.4% citrus pectin and 0.01% marmite. Growth took place at 25° and lasted for 7–10 days.

Preparation of enzyme The cells were centrifuged off, and the enzyme in the supernatant fluid concentrated as follows. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to a final concentration of 40% saturation and the resulting precipitate discarded. Further

$(\text{NH}_4)_2\text{SO}_4$ was then added to give a final concentration of 90% saturation. The precipitate, containing the enzyme, was allowed to rise to the surface of the liquid, and the fluid beneath siphoned off and discarded. The precipitate was collected and dialyzed for 24 hr in collodion sacs against running tap water. The enzyme, now in solution within the dialyzing sac, was precipitated by the addition of 3 vols of methanol at room temperature, spread on a dish, and allowed to dry at 37°. The dry precipitate was powdered in an agate mortar, such preparations of the pectin esterase can be kept indefinitely. For use, the powder was rubbed up in water (30 mg/ml), and the solution brought to pH 7.5 with *N*-NaOH.

Substrate Throughout the work the pectin used was a commercial citrus pectin, which was kindly provided by Chivers and Sons, Ltd, Histon, Cambs. This contained at least 30% invert sugar, the methoxyl content of the powder was estimated by the gravimetric micro Zeisel apparatus and found to be 6.2%. For the enzyme studies a 3% solution, brought to pH 7.8 with *N*-NaOH, was used.

Estimation of activity A manometric technique was used to estimate pectin esterase activity. Since the action of the enzyme liberates acidic groups on de-esterification, the activity can be followed by the evolution of CO_2 from a suitable bicarbonate buffer. The manometer cups contained 0.5 ml of 0.3M NaHCO_3 solution, 0.5 ml enzyme solution, and 1.0 ml pectin solution, and were filled with a N_2/CO_2 gas mixture containing 5% (v/v) CO_2 , at the experimental temperature (37°) this gave a pH of 7.8.

RESULTS

Confirmation that the manometric reaction studied is a de-esterification In order to ascertain that the reaction studied is in fact the hydrolysis of an ester linkage, the relationship between the liberation of

methanol and the production of carboxyl groups (as measured manometrically) was determined For this purpose a series of manometers, each containing 1 0 ml 2 % pectin, 0 5 ml enzyme, and 0 5 ml buffer, were set up At intervals after tipping, the reaction in one manometer was stopped by adding trichloroacetic acid to bring the pH to 1 0 After removing the cup contents, sodium hydroxide was added to bring the pH to 5 0, and the methanol distilled in the Markham (1942) apparatus The methanol in a sample of the distillate was determined colorimetrically by Holden's (1945) modification of a method originally used by Schryver & Wood (1920) Fig 1 shows that the production of methanol runs parallel to the liberation of carboxyl groups

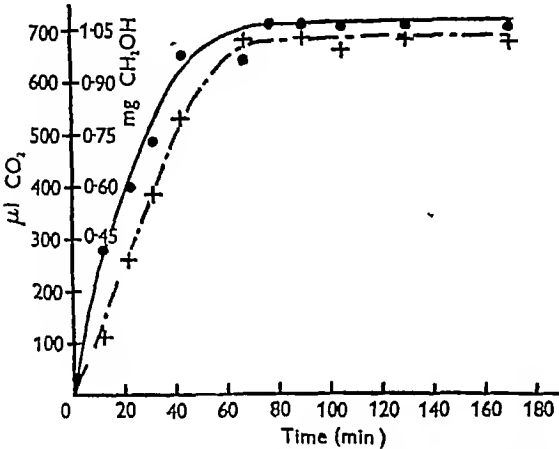


Fig 1 Relationship between methanol production and manometric estimation of CO₂. The manometer cups contained 1 0 ml. 2% Chivers pectin, 0 5 ml enzyme solution and 0 5 ml 0 3M-NaHCO₃. —, Methanol production, — —, CO₂ output

Control experiments, using pectin which had previously been demethylated by cold 0 1 N sodium hydroxide, were set up, and were found to give no gas output The spontaneous demethylation of pectin at pH 7 8 is negligible

Table 1 Relationship between growth medium and production of the adaptive enzyme pectin esterase

Growth medium containing	Enzyme activity
Glucose	—
Galacturonic acid	+
Pectin	+
Pectin, previously demethylated by 0 1 N NaOH	+
Galactose	—

Adaptive nature of the enzyme After subculture into a medium containing no pectin, viz tryptic digest of casein+glucose+marmite, the organism was sown into the same medium, and after 7 days of growth, the procedure for the preparation of the

enzyme followed The resulting powder when rubbed up with water had a negligible demethylating action on pectin When other carbohydrates were substituted for glucose in the growth medium, the enzyme activities found were as shown in Table 1

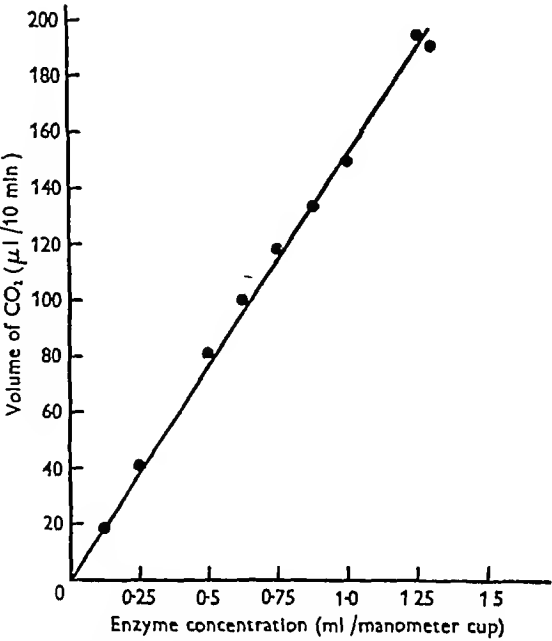


Fig 2 Variation in reaction velocity with enzyme concentration The manometer cups contained 1 0 ml 3% pectin, and 0 5 ml 0 3M NaHCO₃

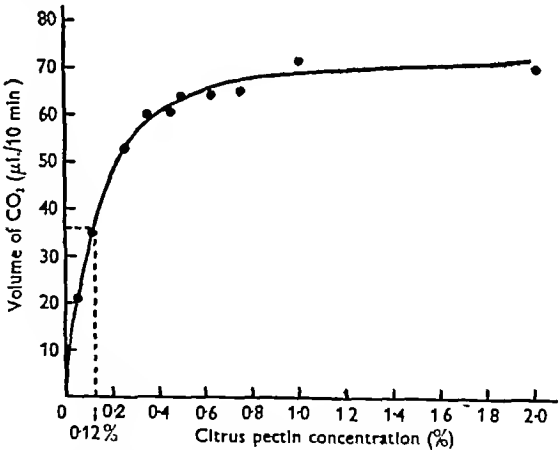


Fig 3 Variation in rate of reaction with concentration of citrus pectin The manometer cups contained 0 5 ml 0 3M NaHCO₃ (in the main cup) and 0 5 ml enzyme solution (side bulb)

Enzyme kinetics

Variation of reaction velocity with enzyme concentration Fig 2 shows that the rate of the reaction is proportional to the enzyme concentration over the range studied

Substrate concentration The variation of the rate of the reaction with the concentration of citrus pectin is shown in Fig 3 The concentration of

pectin required to give a reaction velocity half that of the maximum velocity is 0.12%, and that required to give maximum velocity is 1.0%. These figures represent concentrations of 0.75 mg $\text{OCH}_3/100$ ml and 6.2 mg $\text{OCH}_3/100$ ml respectively.

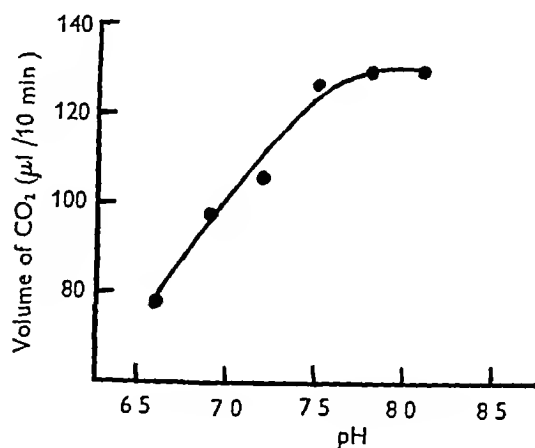


Fig 4 Variation in reaction velocity with pH. The manometer cups contained 1.0 ml 3% pectin and 0.5 ml of the required NaHCO_3 buffer solution (main cup) and 0.5 ml enzyme solution (side bulb).

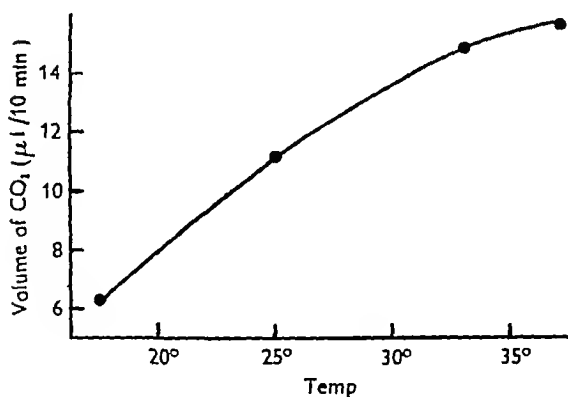


Fig 5 Variation of reaction velocity with temperature. The manometers contained 1.0 ml 3% pectin, 0.5 ml 0.3M- NaHCO_3 (main bulb) and 0.5 ml enzyme (side bulb).

pH effect The use of bicarbonate buffer restricts the manometric method to the pH range from 6.6 to 8.1. The variation of the activity of an enzyme solution, as measured by the initial steady rate of gas output, was investigated over this range, the results are shown in Fig 4. All subsequent experiments were carried out at pH 7.8.

Effect of temperature The activity of the enzyme was tested at four different temperatures. Fig 5 shows the increase of velocity with increasing temperature. The change in pH caused by the change in temperature is so small as to be negligible.

Substrate specificity of the enzyme As the action of the enzyme involves the splitting of an ester linkage, the specificity for various ester links was investigated. Samples of pectins from different sources were all attacked by the enzyme. These included British Drug Houses citrus pectin, '100 grade', with OCH_3 content 4.2% as determined with the gravimetric micro Zeisel apparatus, Hopkins and Williams pectin (source unknown, 4.0% OCH_3), spray-dried apple pectin (2.64% OCH_3) and citrus pectin (6.2% OCH_3).

With each of these pectins it was found that the enzyme split about 75% of the methylated ester linkages (with Hopkins and Williams pectin, 75%, British Drug Houses pectin, 79%, apple pectin, 75%, and Chivers pectin 74%).

The pectin chain consists of 1.4 α -linked galacturonic acid units, the carboxyl groups of which may be esterified with methanol. A sample of the methyl ester of α -methylgalacturonic acid was synthesized and tested as a substrate for the enzyme. Neither this, nor a sample kindly given by Dr J. S. D. Bacon, was split by the pectin esterase at a concentration of 8.5 mg ester/ml. This fact might provide an explanation for the incompleteness of the demethylation by the pectin esterase preparation. The enzyme preparation contains also a pectinase, which breaks the pectin chain to yield reducing material, presumably galacturonic acid or its methyl ester, or degraded fragments. If some methyl ester were in fact produced in the course of a manometric experiment, demethylation of the whole pectin molecule could not be complete, as the ester is not attacked by the pectin esterase. However, more recent work on the pectinase from *Ps. prunicola* has shown that an enzyme solution of similar pectin esterase activity to that used in the above experiments possesses a very feeble pectinase activity under the experimental conditions used, and that this activity could not explain the fact that 25% of the methyl ester links are not broken by the pectin esterase. Since the micro Zeisel apparatus estimates both ethoxyl and methoxyl groups, some of the unbroken ester links may be esterified with ethanol, and the possibility exists that these cannot be attacked by the enzyme.

The enzyme preparation does not attack ethyl acetate or ethyl oxalate, but tributyrin, triacetin, diacetin and monoacetin are all attacked. The di and mono-butyryns were not available for test. The volumes of CO_2 obtained from the four glycerides tested were in each case less than theoretical, viz tributyrin 66%, triacetin 71%, diacetin 67%, monoacetin 75% of theoretical. Addition of further amounts of enzyme after the reaction had ceased caused no further evolution of CO_2 . In order to see if the enzyme catalyzed the back reaction between glycerol and acetic acid, manometers were set up

containing 1 ml 15% glycerol solution, 1.0 ml 0.1 M-sodium acetate, 0.5 ml 0.3 M-bicarbonate solution and 0.5 ml of the enzyme solution. Occurrence of the back reaction would have given rise to an uptake of CO_2 from the system, but in fact no such uptake was observed. Under these conditions, therefore, no back reaction takes place, so that the incompleteness of the de-esterification cannot be due to the existence of an equilibrium between the back and forward reactions.

A crude acetone-powder preparation from liver, which contained an active esterase, had no pectin-esterase activity when tested by the manometric method. Consequently, liver esterase has no pectin-esterase activity under these conditions, although the enzyme preparation from *Ps. prunicola* has an esterase action on glycerol esters. This could, however, be due to the presence of esterases in the crude enzyme preparation, although the non-inhibition by diisopropyl fluorophosphonate does not support this view (see below).

Inhibitors The rate of CO_2 output remained unchanged in the presence of 0.01 M-copper sulphate, potassium cyanide, ferrous sulphate, sodium azide and iodoacetate. A final concentration of 0.0001 M-diisopropyl fluorophosphonate, which was sufficient to cause 100% inhibition of liver esterase, had a negligible effect on the reaction towards pectin and tributyrin. Tannic acid (0.001 M), which is said to inhibit the action of pectinases (Kertesz, 1936), caused no inhibition of the pectin esterase reaction. The enzyme and pectin solutions were submitted to

dialysis against tap water and then distilled water, and the activity determined in the presence and absence of sodium chloride and sodium oxalate, which have been reported as being necessary for activation of the enzyme from tomatoes (Hills & Mottern, 1947). The velocity of the reaction was increased 20% by 0.05 M-sodium chloride, but was unaffected by 0.002 M-oxalate.

Further work is being carried out on the pectinase enzymes produced by *Ps. prunicola*, and on the separation of the pectin esterase and pectinase enzymes.

SUMMARY

1 A manometric method is described for the determination of pectin-esterase activity, and has been used to investigate some of the properties of such an enzyme obtained from *Ps. prunicola*.

2 The exo-enzyme, produced by *Ps. prunicola*, is formed adaptively in response to growth in the presence of pectin, pectate, and galacturonic acid.

3 The enzyme preparation splits off 75% of the methoxyl groups (estimated by the micro-Zeisel apparatus) of various pectins, and also hydrolyzes tributyrin, triacetin, diacetin and monoacetin, to the extent of approximately 70%. It has no action on ethyl acetate, ethyl oxalate or the methyl ester of α -methylgalacturonic acid.

I wish to express my thanks to Dr M Stephenson, F.R.S., Dr R Hill, F.R.S., Dr E F Gale and Dr W J Dowson for their interest and advice, and to the Agricultural Research Council for a personal grant.

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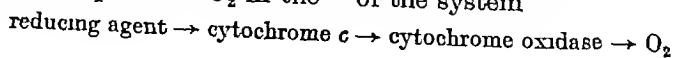
The Measurement of the Cytochrome Oxidase Activity of Enzyme Preparations

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Cytochrome oxidase has been defined by Keilin & Hartree (1938) as the enzyme responsible for the oxidation of reduced cytochrome *c*. The activities of oxidizing enzymes are usually measured by determining the rate of O_2 uptake in the presence of excess substrate. This method is, however, scarcely applicable in the case of cytochrome oxidase since the amount of reduced cytochrome *c* required to give a measurable uptake of O_2 in the

types of manometers usually employed is too great (thus 3.3 ml of 0.1% reduced cytochrome *c* requires only 1.1 μl O_2 for complete oxidation). The rate of oxidation of cytochrome *c* can be measured spectrophotometrically (e.g. Albaum, Tepperman & Bodansky, 1946), but the apparatus required is not always available. Consequently an indirect method is usually employed in which the rate of O_2 uptake of the system



(the arrows indicating the direction of hydrogen or electron transfer) is measured. It is generally believed that, under the conditions of the experiment, cytochrome *c* is reduced by the reducing agent as soon as it is oxidized by the cytochrome oxidase oxygen, and so the rate of O₂ uptake is a measure of the cytochrome oxidase activity. This method has frequently been employed in the study of the action of inhibitors on the cytochrome oxidase system, and it is often assumed, without further examination, that any decrease in the rate of O₂ uptake is due to inhibition of the enzyme.

It has been shown (Slater, 1948*a*, 1949*a*) that treatment of heart-muscle preparation with BAL (2,3-dimercaptopropanol) causes the complete inactivation of the succinic oxidase system. As cytochrome oxidase is a part of this system, the effect of BAL on this enzyme was also studied. It was found that there was little if any inhibition when ascorbic acid or hydroquinone was used as the reducing agent in the measurement of the cytochrome oxidase activity, but a considerable inhibition (30–40%) if *p*-phenylenediamine was the reducing agent. To investigate this further, a study has been made of the factors involved in the measurement of cytochrome oxidase activity.

The present paper reports the results of this study. Under the conditions usually employed in making this measurement, the rate of O₂ uptake was found to depend not only on the cytochrome oxidase activity, but also on the concentrations of cytochrome *c* and reducing agent, and on factors which affect the catalytic activity of the cytochrome *c*. A procedure suitable for studying the true activity of cytochrome oxidase, independently of these factors, is described. A preliminary account of some of the findings of this investigation has appeared elsewhere (Slater, 1948*b*).

METHODS

Cytochrome oxidase. The heart-muscle preparation of Keilin & Hartree (1947*b*) was used.

Cytochrome c, containing 0.34% Fe, was prepared by the method of Keilin & Hartree (1945). The solution contained 6.7 × 10⁻⁴ M cytochrome *c* in 0.5% NaCl.

Phosphate buffer. A Sorensen phosphate buffer, pH 7.3, was used.

Manometric experiments were carried out in Barcroft differential manometers at 38°. The reducing agent was added (by displacement of a dangling tube) at zero time, after temperature equilibration. Readings were begun 3 or 5 min after the addition. In all experiments the gas phase was air.

RESULTS

Reducing agents

Table 1 lists various reducing agents commonly employed for the manometric estimation of cytochrome oxidase activity, together with their

Table 1 *Capacity of various reducing agents to reduce cytochrome c*

(*E*₀' of cytochrome *c* = 0.262 V at pH 7.3, 30° (Stotz, Sidwell & Hogness, 1938*a*). Initial concentrations: reducing agent, 0.05 M; oxidized cyt *c*, 6 × 10⁻⁵ M. The figures in the third column correspond to the equilibrium state after the reducing agent has been oxidized by the absorption of 100 μl O₂. Total vol. 3.3 ml.)

Reducing agent	<i>E</i> ₀ ' at pH 7.3, 30° (V)	Cyt <i>c</i> in reduced form (%)
Ascorbic acid	0.049 (Ball, 1937)	99.9
Hydroquinone	0.255	84.4
Catechol	0.354 (calculated from Ball & Chen, 1933)	9.4
Adrenaline	0.364 (calculated from Ball & Clark, 1931)	7.6

oxidation-reduction potentials at pH 7.3. It also shows the proportion of cytochrome *c* in the reduced form, at equilibrium, when the initial concentration of reducing agent was 0.05 M, that of oxidized cytochrome *c* was 6 × 10⁻⁵ M, and the reducing agent has been oxidized by the absorption of 100 μl O₂, the total volume of the solution being 3.3 ml. It is obvious that catechol and adrenaline are not satisfactory reducing agents. It was found, in fact, that the rate of O₂ uptake, corrected for blank (see p. 307), of 0.05 M-catechol, 6 × 10⁻⁵ M-cytochrome *c* and enzyme preparation, was only 21% that of the similarly corrected figure obtained for the same concentration of hydroquinone in the presence of the same concentration of cytochrome *c* and the same enzyme preparation. Unfortunately, the oxidation-reduction potential of *p*-phenylenediamine, one of the most useful reagents for the determination of cytochrome oxidase activity, cannot be determined at pH 7.3. Thus Clark, Cohen & Gibbs (1926) stated that 'attempts to measure the system by which *p*-phenylenediamine is the reductant were frustrated by the extreme instability of the system'. Fieser (1930) reported no success with measurements in solutions less acid than pH 5. Barron (1939) calculated, by extrapolation of the data of Fieser at acid reactions, that the *E*₀' at pH 7.0 of *p*-phenylenediamine would be +0.381 V, i.e. in the same region as catechol and adrenaline. Stotz, Sidwell & Hogness (1938*b*), on the other hand, state that *p*-phenylenediamine possesses a much lower potential than hydroquinone and is even capable of reducing cytochrome *b* (*E*₀' = -0.04 V). However, neither Borel (1945) nor the author has been able to confirm this reduction of cytochrome *b*.

It must be emphasized that the figures given in Table 1 are theoretical, calculated from the oxidation-reduction potentials, assuming a simple oxidation of the reducing agent, without further reaction of the oxidized form. Ball & Chen (1933)

pointed out that the substance with the higher oxidation-reduction potential can reduce that with the lower if the oxidized state of the former is unstable. For example, adrenaline can reduce 2,6-dichlorophenolindophenol at pH 7, although originally only about 0.1% of the dye would be reduced, and the equivalent amount of adrenaline oxidized.

Boss & Friedenwald (1946) reported that cytochrome *c* had no effect on the oxidation. These discrepancies are possibly due to differences in the amounts of impurities in the cytochrome *c* preparations used in the different investigations. The cytochrome *c*-catalyzed oxidation of ascorbic acid is probably not inhibited by heart muscle preparation.

Table 2 Rates of oxidation of reducing agents in the absence of enzyme
(Phosphate buffer, 0.15M, pH 7.3, total vol. 3.3 ml)

Reducing agent	Concentration (M)	Initial rate of O ₂ uptake (μl/hr)				'Blank' obtained by extrapolation* in presence of 6 × 10 ⁻⁵ M-cyt c
		Cyt c absent	With 2 × 10 ⁻⁵ M-cyt c	With 4 × 10 ⁻⁵ M-cyt c	With 6 × 10 ⁻⁵ M-cyt c	
Hydroquinone	0.017	107	—	—	125	—
	0.05	294	—	—	303	300
Catechol	0.017	42	—	—	51	—
	0.05	177	—	—	193	196
<i>p</i> -Phenylenediamine	0.05†	27	—	—	43	27
Ascorbic acid	0.0017	24	—	—	16	—
	0.0085	89	—	—	53	—
	0.017	122	—	—	81	76
	0.026	156‡	91	93	101	—

* See Fig. 1 and text, p. 308
† Correction for lower concentrations is negligible
‡ Rate of O₂ uptake in presence of heart muscle preparation (0.3 mg dry wt/ml) = 80 μl/hr

The oxidized adrenaline quickly decomposes and, in order to restore the equilibrium, more of the dye must be reduced. In this way the reduction of the dye can proceed to completion. A similar situation may exist with respect to the reduction of cytochrome *c* by *p*-phenylenediamine or adrenaline. The reaction with adrenaline is very complex (see p. 308).

The reducing agents listed in Table 2 are oxidized to a certain extent in the absence of the enzyme. This so-called 'auto-oxidation' is due to catalysis by traces of heavy metals present in the reagents, and its magnitude depends on the purity of the reagents and on the pH. It must be determined as a 'blank' and subtracted from the total O₂ uptake in order to obtain a measure of the cytochrome oxidase activity. However, this 'blank' may not be the same in the presence of heart-muscle preparation as in the buffer solution alone, e.g. the oxidation of ascorbic acid, which is catalyzed by traces of heavy metals (Barron, De Meio & Klemperer, 1935-6), is inhibited by heart-muscle preparation (see footnote to Table 2, and also Borei, 1945), probably because —SH groups in the latter react with traces of Cu⁺⁺ present in the solution. Table 2 shows that it is also inhibited by small concentrations of cytochrome *c*, although larger concentrations of the latter catalyze the oxidation. Borei (1945) reported catalysis of the oxidation of ascorbic acid by cytochrome *c*, but did not find any inhibition by small concentrations, Herrmann,

since, according to Borei (1945), it is not affected by diethyldithiocarbamate, a copper-

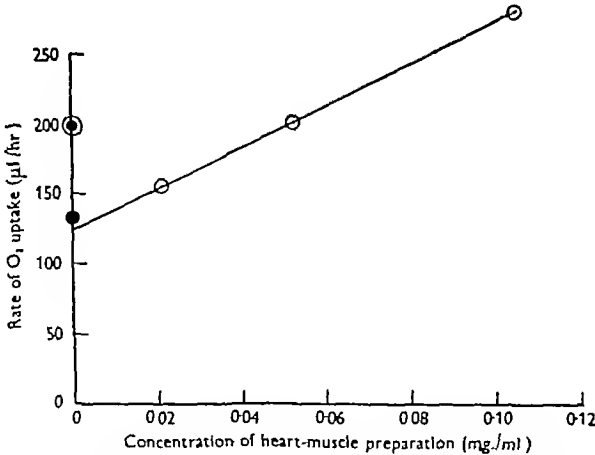


Fig. 1 Method of calculating 'blank' by extrapolation to zero enzyme concentration. 0.017M Ascorbic acid (neutralized), 0.15M-phosphate buffer, vol. 3.3 ml. Ascorbic acid introduced from dangling tube after equilibration. ○, rate of oxidation in presence of 6 × 10⁻⁵M cytochrome *c* and varying amounts of heart-muscle preparation, ●, 6 × 10⁻⁵M-cytochrome *c*, no enzyme, ⊙, no cytochrome or enzyme. (Note that the higher rates of O₂ uptake compared with the figures given in Table 2 are due to the use of a different phosphate buffer.)

binding reagent which strongly inhibits the copper-catalyzed oxidation of ascorbic acid.

Probably the best method of determining the true 'blank' oxidation is that of Schneider & Potter (1943), in which the rates of O₂ uptake in the presence of a constant concentration of reducing agent and cytochrome *c*, but with varying concentrations of enzyme, are extrapolated to zero enzyme concentration. An example of this method is shown in Fig 1, and the values obtained are included in Table 2. With all four of the reducing agents studied, these values are only slightly different from those obtained by direct measurement in the presence of cytochrome *c* and in the absence of enzyme. All measurements reported in this paper have been corrected for the blank.

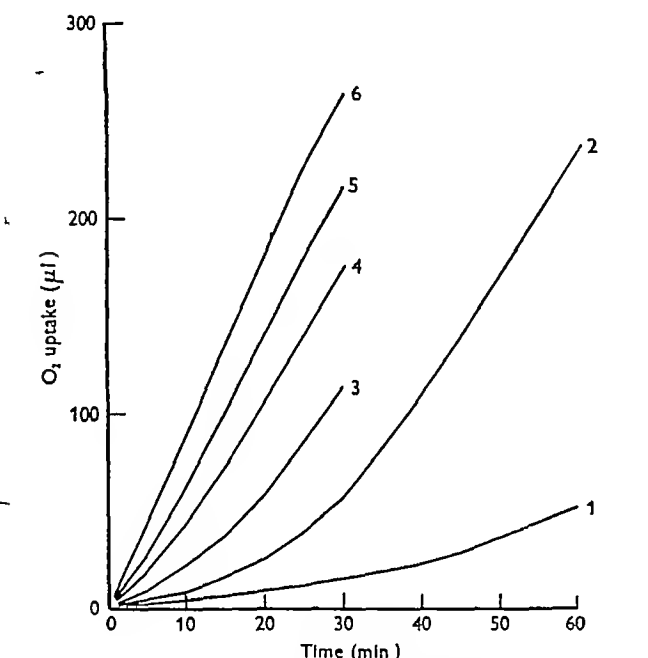


Fig 2 O₂ uptake of adrenaline. 0.15M Phosphate buffer, 3.3 ml total vol. 10 mg solid adrenaline added at (zero-5) min. by dislodging dangling tube, manometric measurements commenced at zero time.

Curve	Concentration of cytochrome <i>c</i> (M)	Concentration of heart-muscle preparation (mg fat free dry wt/ml)
1	0	0
2	6×10^{-5}	0
3	6×10^{-5}	0.019
4	6×10^{-5}	0.07
5	6×10^{-5}	0.14
6	6×10^{-5}	0.28

Adrenaline is only slowly oxidized in the absence of cytochrome *c* or of cytochrome oxidase (Fig 2). The oxidation is, however, strongly catalyzed by cytochrome *c*, and the course of this reaction shows that the oxidation products of adrenaline strongly catalyze the latter's oxidation at a rate which masks that due to the cytochrome oxidase system, when the latter is added. Thus, addition of cytochrome oxidase decreases the initial lag of the oxidation

which occurs in the presence of cytochrome *c* alone, but does not appreciably increase the maximum rate of oxidation. This is clearly seen in Fig 3, where the uptakes in the first 5 min. and the

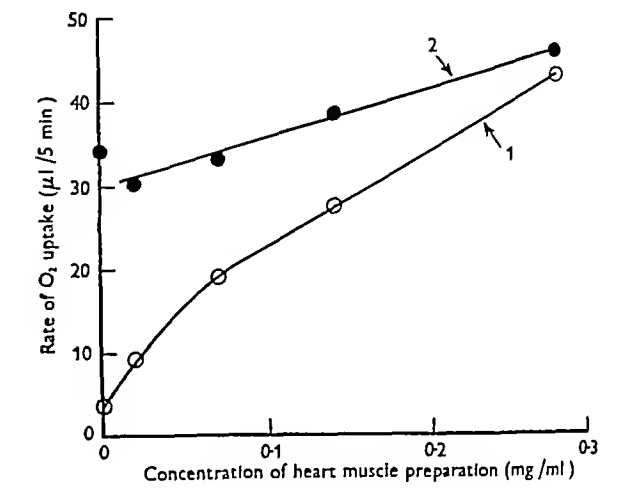


Fig 3 Rate of O₂ uptake of adrenaline in the presence of different concentrations of heart-muscle preparation (calculated from Fig 2). 6×10^{-5} M cytochrome *c*, 0.15M phosphate, total vol. 3.3 ml, 10 mg adrenaline. Curve 1, oxygen uptake between zero time (5 min. after adding adrenaline) and 5 min.; curve 2, maximum rate of O₂ uptake.

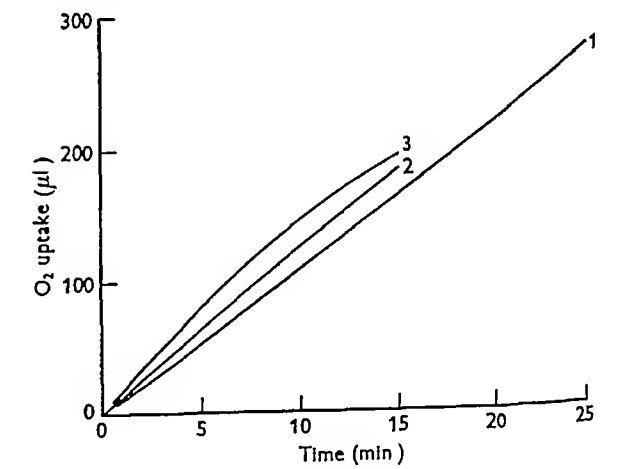


Fig 4 Course of the O₂ uptake of 0.017M neutralized ascorbic acid (curve 1), 0.05M *p*-phenylenediamine (curve 2), 0.05M hydroquinone (curve 3), each in the presence of 6×10^{-5} M cytochrome *c* and heart-muscle preparation (0.28 mg/ml). Total vol. 3.3 ml, 0.15M phosphate buffer. Reducing agents added at (zero-5) min.

maximum rates of O₂ uptake are plotted against the enzyme concentration. Thus, although in the presence of cytochrome *c* and enzyme, adrenaline, after an initial lag, shows a constant rate of O₂ uptake, this measurement is useless for the measurement of cytochrome oxidase activity. The fact that this rate of O₂ uptake is about the same as that obtained with other reducing agents in the presence

of cytochrome *c* and the amount of heart muscle preparation usually employed in these studies is purely a coincidence, it is the reason why this effect of cytochrome *c* and the oxidation products of adrenaline on the latter's oxidation has not previously been observed

The course of the O_2 uptake with the other reducing agents used is shown in Fig 4. In the case of hydroquinone and *p*-phenylenediamine, the rate of O_2 uptake decreased with time, and the initial rate, obtained by extrapolation to zero time, was used as the measure of activity. This decrease with time is presumably due to the toxic action of the reducing agent or of its oxidation product. Ascorbic acid showed a slight lag, and the maximum uptake was obtained 10–15 min after the addition, and was then constant for the next 10–15 min. This constant maximum rate was used as the measure of activity.

Effect of cytochrome *c* concentration

As has been pointed out by Keilin & Hartree (1940), the amount of cytochrome *c* in the heart-muscle preparation is nearly sufficient to oxidize succinate at the maximal rate, the addition of a large excess of cytochrome *c* increases the rate of oxidation of succinate by only about 20%. This shows that the endogenous cytochrome *c* of the heart-muscle preparation can be rapidly reduced by succinate (through intermediate carriers) and, more important in the present connexion, can be rapidly oxidized by the cytochrome oxidase. Keilin & Hartree (1938) showed, however, that, with the exception of *p*-phenylenediamine, none of the reducing agents studied was oxidized by heart-muscle preparation at an appreciable rate, unless cytochrome *c* was added. Since the oxidation of cytochrome *c* is common to both the oxidation of succinate and of these reducing agents, the difference between the rates of oxidation of succinate and of the reducing agents must lie in the relative rates of reduction of the endogenous cytochrome *c*. It is interesting to note, in this connexion, that the reduction by succinate is an enzymic reaction, while that by ascorbic acid is non enzymic.

The rate of reduction of the endogenous cytochrome *c* of the heart muscle preparation by 0.025 M-ascorbic acid was determined under completely anaerobic conditions in the presence of 0.01 M KCN. The heart-muscle preparation contained 2.3×10^{-5} M cytochrome *c* (Slater, 1949b), i.e. 2.3×10^{-2} micromole/ml and this was half reduced in about 25 sec at 20°. If we assume that the reduction of cytochrome *c* is monomolecular it can be calculated that the initial rate of reduction is 3.8×10^{-2} micromole cytochrome *c*/min/ml of heart-muscle preparation. Such a reaction would cause the uptake of 13 μ l O_2 /hr/ml of heart-muscle

preparation. Owing to the low concentration of the cytochromes and their high catalytic activity, spectroscopic observations are usually made with the undiluted heart-muscle preparation, while only 0.02 ml of the preparation is taken in the manometric experiments. Thus the rate of O_2 uptake in the manometric experiments due to this reaction would be $0.02 \times 13 = 0.26 \mu$ l/hr, which is negligible even when allowance is made for the higher temperature in the manometric experiments. Under the same conditions, 0.05 M-*p*-phenylenediamine reduced the endogenous cytochrome *c* almost instantaneously (<5 sec).

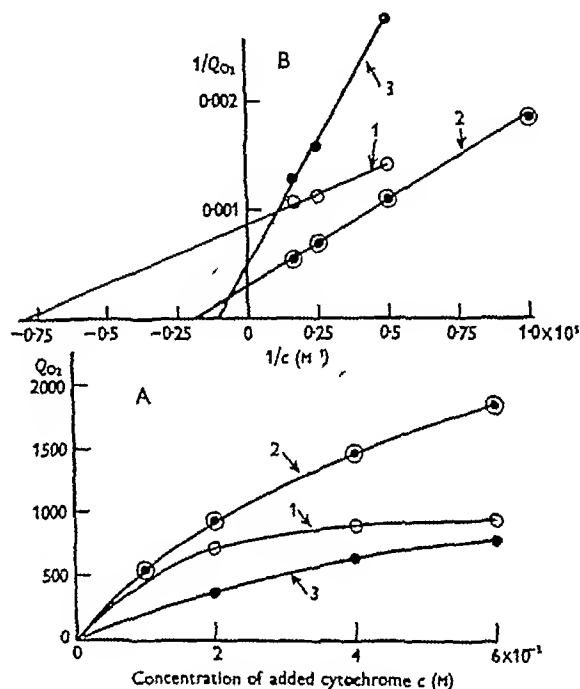


Fig 5 A, effect of concentration of cytochrome *c* on the rate of oxidation of ascorbic acid in the presence of heart-muscle preparation. Ascorbic acid, 0.025 M, NaCl, 0.0008 M, 0.51 mg heart-muscle preparation (wt of fat-free dried material), total vol 3.3 ml. Curve 1, 0.01 M-phosphate buffer, curve 2, 0.065 M-phosphate buffer, curve 3, 0.146 M-phosphate buffer. B, figures in A, plotted according to the procedure of Lineweaver & Burk (1934).

The rate of reduction of the cytochrome *c* of the heart-muscle preparation by ascorbic acid is much less than that of the same concentration of pure cytochrome *c*, which was reduced almost instantaneously. In this respect, ascorbic acid behaves in the same way as cysteine, already studied by Keilin (1930).

The effect of the concentration of cytochrome *c* on the rate of O_2 uptake of ascorbic acid (corrected for 'blank') in the presence of heart-muscle preparation at different concentrations of phosphate buffer is shown in Fig 5A. In Fig 5B these figures have been plotted according to the procedure of

Lineweaver & Burk (1934), i.e. the inverse of the activity against the inverse of the cytochrome *c* concentration. In Fig 6A, B, similar data are given for *p*-phenylenediamine oxidation in 0.15 M-phosphate. The difference between ascorbic acid and *p*-phenylenediamine is most clearly seen in Fig 7, which shows the result of parallel experiments obtained with the same heart muscle preparation in 0.065 M phosphate.

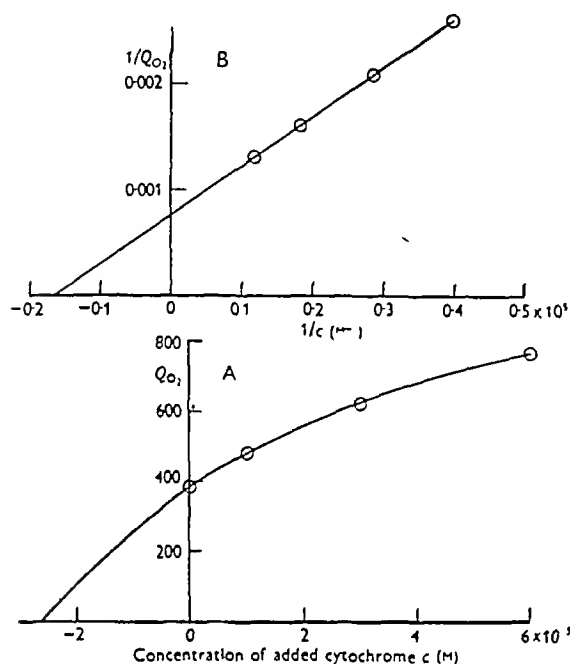


Fig 6 A, effect of concentration of cytochrome *c* on the rate of oxidation of *p*-phenylenediamine in the presence of heart-muscle preparation. *p*-Phenylenediamine, 0.05 M, phosphate buffer, 0.15 M, 1.02 mg heart-muscle preparation (wt of fat-free dried material), total vol 3.3 ml. The curve has been extrapolated to the abscissa by assuming that the points fall on a rectangular hyperbola. B, figures in A, plotted according to the procedure of Lineweaver & Burk (1934).

The endogenous cytochrome *c*, which can be reduced rapidly by *p*-phenylenediamine but not by ascorbic acid, is much more active catalytically than added cytochrome *c*. Thus, by the extrapolation shown in Fig 6A, it was found that in 0.15 M-phosphate, the endogenous cytochrome *c* had a catalytic activity equal to that of 2.5×10^{-6} M added cytochrome *c*. The actual concentration of endogenous cytochrome *c* was 2.8×10^{-7} M, i.e. the endogenous cytochrome *c* was about 100 times as active as that in solution (cf Keilin, 1930, Keilin & Hartree, 1940, 1945). The corresponding ratio of activities in 0.065 M phosphate, calculated from Fig 7, was 40. It is not surprising that the endogenous cytochrome *c* should be so much more active than that added, since the cytochrome *c* is firmly bound to the particles of the heart-muscle prepara-

tion is probably in the most favourable position for the transfer of electrons to cytochrome oxidase.

The relative slopes of the two curves in Fig 7B show that when *p*-phenylenediamine is the reducing agent, not only is the endogenous cytochrome *c* more effective than is the case when ascorbic acid is used as a reducing agent, but added cytochrome *c* is also more effective*. It is important to note, however, that the activity at infinite cytochrome *c* concentration is independent of the reducing agent.

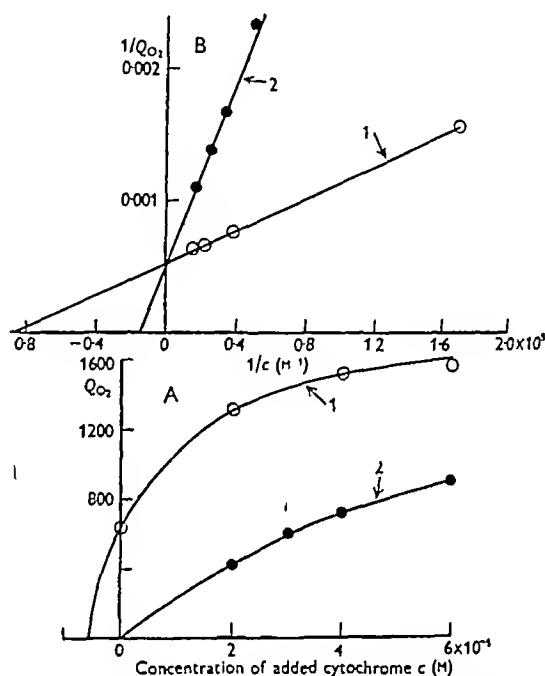


Fig 7 A, effect of different concentrations of cytochrome *c* on the rate of oxidation of ascorbic acid and *p*-phenylenediamine. Phosphate buffer, 0.065 M, 0.41 mg (fat-free dry wt) heart muscle preparation, total vol 3.3 ml. Curve 1, *p*-phenylenediamine (0.05 M), extrapolated as in Fig 6A, curve 2, ascorbic acid (0.025 M). B, figures in A, plotted as in Figs 5A and 6A.

These experiments show that very large concentrations of cytochrome *c* are required for maximal activity of the cytochrome oxidase, especially when ascorbic acid is the reducing agent and high concentrations of phosphate buffer are employed. Thus it can be calculated from Fig 5B that, in 0.146 M phosphate, 10^{-3} M-cytochrome *c* ($=16$ mg/ml) is required to give 90% of the maximum activity. This is about 100 times the concentration of total protein contributed by the heart-muscle preparation itself, and obviously it would be quite impossible to have such concentrations of cytochrome *c* in the cell itself. This finding is another example of

* This effect of reducing agent on the activity of the added cytochrome *c* is much more marked at 0.065 M phosphate than at 0.15 M.

the poor catalytic activity of cytochrome *c* in solution compared with that in the cell. In 0.01M-phosphate, 1.2×10^{-4} M-cytochrome *c* is necessary for 90% activity. It follows that it is impossible to measure directly the full activity of the cytochrome oxidase present in 0.5 mg heart-muscle preparation by determining the rate of oxygen uptake in the presence of excess cytochrome *c*. This can be done only by measuring the activity at different cytochrome *c* concentrations and extrapolating to infinite concentration. The concentrations of cytochrome *c* found in the present study to be necessary for maximal activity of the cytochrome oxidase are very much greater (about 10 times) than those found by Stotz, Altschul & Hogness (1938) and Borel (1945). This is probably due to the much greater activity of the heart-muscle preparation used in the present study. The inhibitory effect of high cytochrome *c* concentrations on the oxidation of *p*-phenylenediamine by heart-muscle preparation, reported by Borel, was not found in the present study.

Effect of phosphate concentration

The effect of the concentration of the phosphate buffer on the rate of oxidation of ascorbic acid in the presence of a fixed concentration of cytochrome *c* and ascorbic acid is shown in Fig. 8. The amount of ascorbic acid used was the same as that in Fig. 5, and the amount of cytochrome *c* was the highest concentration used in Fig. 5, the same enzyme preparation was used. From Fig. 5B, the maximum activity (i.e. at infinite cytochrome *c* concentration) can be calculated from the point at which each straight line intersects the ordinate, which equals the inverse of the maximum activity. The point at which the line intersects the abscissa equals $-1/(\text{cyt } c)_{\frac{1}{2}}$, where $(\text{cyt } c)_{\frac{1}{2}}$ is the concentration of cytochrome *c* required for half maximal activity. These quantities, calculated from Fig. 5B, are given in Table 3, which also includes the activities in the presence of 6×10^{-5} M-cytochrome *c*. The difference in the slopes of the lines in Fig. 5B at different

phosphate concentrations shows how dangerous it is to measure the activity at only one cytochrome *c* concentration. Thus, at all concentrations of cytochrome *c* shown in Fig. 5, the activity in 0.146M-phosphate was less than that in 0.01M-phosphate, but the curves show that, if excess cytochrome *c* was used, the activity of the cytochrome oxidase at 0.146M-phosphate would be nearly double that at 0.01M-phosphate (Table 3). In order to measure the true activity of the cytochrome oxidase it is necessary to measure the activity at different cytochrome *c* concentrations and extrapolate to infinite cytochrome *c* concentration.

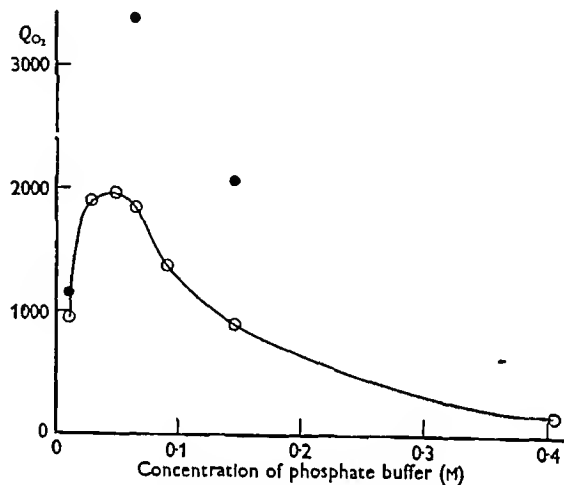


Fig. 8 Effect of phosphate concentration on rate of oxidation of ascorbic acid (0.025M) in the presence of cytochrome *c* (6×10^{-5} M) and heart-muscle preparation (0.154 mg fat-free dry wt/ml), total vol. 3.3 ml. O, activities measured under these conditions; ●, activities at infinite cytochrome *c* concentration, calculated from Fig. 5B.

Keilin & Hartree (1949) (see also Slater, 1949c) have shown that the activity of the succinic oxidase system of the heart muscle preparation is dependent on the concentration of the phosphate buffer used. Figs. 5 and 8 show that the concentration of the

Table 3 Effect of phosphate concentration on the cytochrome oxidase activity of heart-muscle preparation

(Q_{o2} measured at 38°, based on wt. of fat-free dried material)
(Cyt *c*)_½ = concentration of cytochrome *c* required for half maximal activity

Phosphate concentration (M)	Q _{o2}		$\frac{B}{A} \times 100$	(Cyt <i>c</i>) _½ (M)
	At infinite cyt <i>c</i> concentration (A)	In presence of 6×10^{-5} M-cyt <i>c</i> (B)		
0.01	1140	950	83	1.25×10^{-5}
0.065	3360	1840	55	5.4×10^{-5}
0.146	2040	890	44	9.3×10^{-5}

buffer is also very critical so far as the cytochrome oxidase activity is concerned. When ascorbic acid is the reducing agent, the concentration of phosphate buffer has two separate effects (1) on the activity of the cytochrome oxidase itself, measured at infinite cytochrome *c* concentration, the concentration of phosphate buffer which gives optimal activity is 0.065M, (2) on the catalytic activity of added cytochrome *c*, this catalytic activity may be measured either by the inverse of the concentration of cytochrome *c* required for half-optimal activity or by the inverse of the slopes of the straight lines in Fig 5B. The catalytic activity of added cytochrome *c* decreases rapidly with increase of phosphate concentration, and is optimal at low concentrations. Because the optimal phosphate concentration for this second effect is different from that required for the first, it follows that, as has already been mentioned, the effect of phosphate concentration depends upon the amount of cytochrome *c* used.

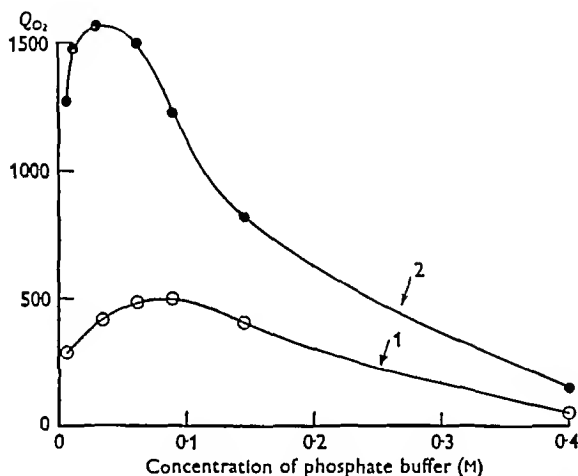


Fig 9 Effect of phosphate concentration on the rate of oxidation of *p*-phenylenediamine (0.05M) by heart muscle preparation in the absence of cytochrome *c* (curve 1) and in the presence of 6×10^{-5} M cytochrome *c* (curve 2). 0.29 mg fat-free dry wt heart muscle preparation/ml, total vol 3.3 ml.

When *p*-phenylenediamine is the reducing agent, in the absence of added cytochrome *c*, the second effect is no longer operative, but the effect of phosphate on the catalytic activity of the endogenous cytochrome *c* must be considered. In this case, this factor is probably all important, except perhaps at high phosphate concentrations, since the system operates far below its full cytochrome oxidase activity. The effect of phosphate concentration on the rate of oxidation of *p*-phenylenediamine by heart-muscle preparation, both in the presence and absence of cytochrome *c*, is shown in Fig 9. In the absence of added cytochrome *c*, the optimum

phosphate concentration is 0.08M, and the curve is much flatter than those already considered. In the presence of added cytochrome *c* (6×10^{-5} M) the curve is similar to that obtained with ascorbic acid.

Keilin & Hartree (1949) found that the addition of denatured globin to heart muscle preparation caused a considerable increase in the succinic oxidase activity, especially at low phosphate concentrations, in fact, the inhibiting effect of low phosphate concentrations is completely removed by the addition of globin. It was found in the present study, however, that denatured globin had little effect (it actually caused a slight inhibition) on the rate of oxidation of *p*-phenylenediamine by heart muscle preparation at any of the phosphate concentrations studied, either in the presence or absence of cytochrome *c*.

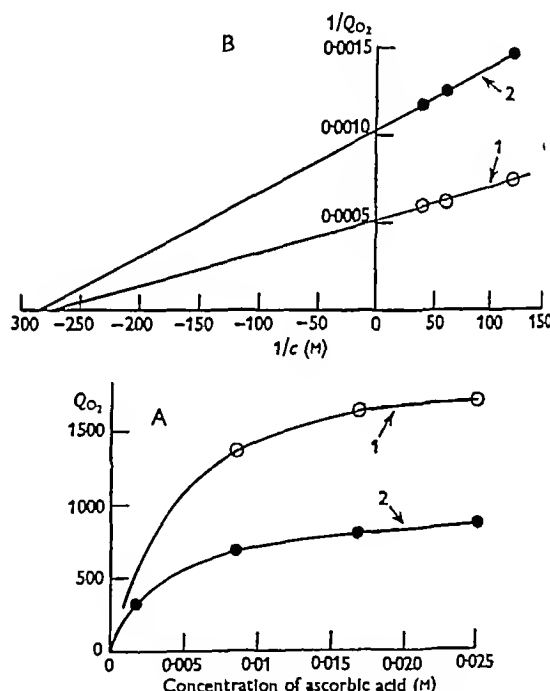


Fig 10 A, effect of ascorbic acid concentration on the rate of its oxidation by heart-muscle preparation (0.15 mg fat-free dry wt/ml) in the presence of cytochrome *c* (6×10^{-5} M), total vol 3.3 ml. Curve 1, 0.065M phosphate; curve 2, 0.146M phosphate. Same heart-muscle preparation as Figs 5, 6. B, figures in A, plotted according to the procedure of Laneweaver & Burk (1934).

Effect of concentration of reducing agent

The effect of the concentration of ascorbic acid on the rate of O_2 uptake at two phosphate concentrations is shown in Fig 10. The activity (Q_{O_2}) at infinite ascorbic acid concentration is 980 in 0.146M-phosphate and 1990 in 0.065M-phosphate. The effect of different concentrations of *p*-phenylene

The effect of two different enzyme concentrations on the rate of oxidation of ascorbic acid in the presence of different amounts of cytochrome c is shown in Fig 12

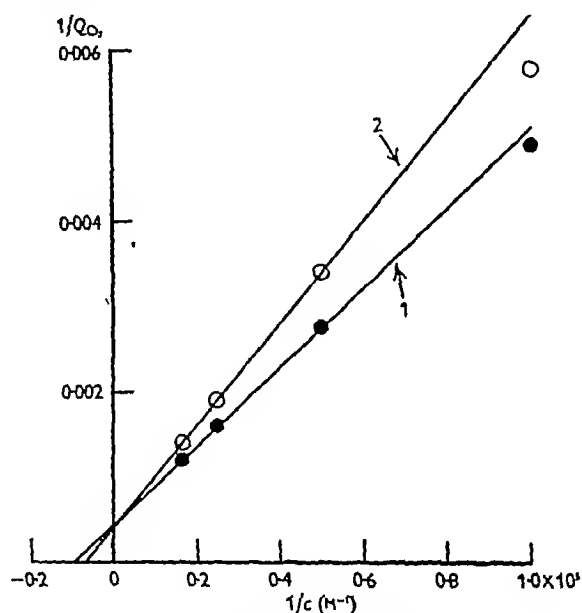


Fig 12 Effect of concentration of heart-muscle preparation on the rate of oxidation of ascorbic acid (0.025M) in the presence of different concentrations of cytochrome c, plotted according to the procedure of Lineweaver & Burk (1934). Phosphate, 0.146M, total vol. 3.3 ml. Curve 1, 0.18 mg (fat free dry wt) heart-muscle preparation/ml, curve 2, 0.36 mg heart-muscle preparation/ml.

Recommended procedure for measuring cytochrome oxidase activity

As a result of the experiments described above, the following procedure for measuring the cytochrome oxidase activity of the Keilm & Hartree (1947b) preparation has been adopted. This procedure should not be used with other preparations.

[illegible]

without first examining the effect of the various factors influencing the rate of O₂ uptake

One complete estimation requires eight manometers, four of these are, however, used for determining the 'blank' oxidation at each level of cytochrome *c*, and these measurements need only be made once for each batch of phosphate buffer and cytochrome *c* The right-hand flasks of the differential manometers are filled as shown in Table 4 The left-hand flasks each contain 3.3 ml 0.065 M-phosphate buffer, pH 7.3 KOH papers are not used

The manometers, with flasks attached, are placed in a bath at 38° and gently shaken for 15–25 min for temperature equilibration It is important that this shaking should not be too prolonged as it causes inactivation of the enzyme The dangling tubes are then dislodged, and the manometers rapidly shaken (at about 140 shakes/min) Readings are taken every 5 min for 35 min The maximum rate of O₂ uptake, which usually occurs 10–15 min after adding the reducing agent and is maintained for a further 10–15 min, is calculated If the maximum rates from the eight manometers are *R*₁, *R*₂, ..., *R*₈, the activities (*v*) may be calculated as shown in Table 5

in which Fe⁺⁺⁺ cyt *c* and Fe⁺⁺ cyt *c* are the oxidized and reduced forms of cytochrome *c* Reaction (1) is the non-enzymic reduction of cytochrome *c*, and reaction (2) is the enzymic oxidation

Cytochrome oxidase preparations, to which cytochrome *c* has been added, will catalyze the oxidation of any substance which can reduce cytochrome *c* sufficiently rapidly Keilin (1930) showed that cysteine is such a substance, and other thiols would be expected to behave similarly This has, indeed, been shown to be the case with the dithiol, 2,3-dimercaptopropanol (Webb & van Heyningen, 1947) and with sodium diethyldithiocarbamate (Keilin & Hartree, 1940) Ames & Elvehjem (1945) have found that cytochrome *c*+kidney homogenate catalyzes the oxidation of glutathione, but believe that this is due to the presence of a glutathione dehydrogenase Since pure glutathione rapidly reduces pure cytochrome *c*, there does not seem any need to postulate the existence of such a dehydrogenase In a later paper, Ames, Ziegenhagen & Elvehjem (1946) claim to have shown that inhibitors act differently on the systems involved in the oxidation of glutathione and ascorbic acid respectively This question requires further investigation, since Ames *et al* measured the rate of oxidation of

Table 5 Calculation of cytochrome oxidase activities

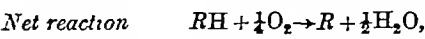
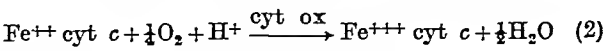
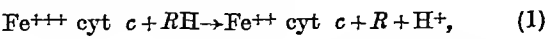
(*c*=cytochrome *c* concentration (M), *v*=cytochrome oxidase activity (corrected))

(<i>c</i>) (M)	1/ <i>c</i> (M ⁻¹)	Activity (uncorr)	Blank	Activity (corr) (<i>v</i>)	1/ <i>v</i>
2 × 10 ⁻⁵	0.5 × 10 ⁵	<i>R</i> ₁	<i>R</i> ₅	<i>R</i> ₁ − <i>R</i> ₅	1/(<i>R</i> ₁ − <i>R</i> ₅)
3 × 10 ⁻⁵	0.33 × 10 ⁵	<i>R</i> ₂	<i>R</i> ₆	<i>R</i> ₂ − <i>R</i> ₆	1/(<i>R</i> ₂ − <i>R</i> ₆)
4 × 10 ⁻⁵	0.25 × 10 ⁵	<i>R</i> ₃	<i>R</i> ₇	<i>R</i> ₃ − <i>R</i> ₇	1/(<i>R</i> ₃ − <i>R</i> ₇)
6 × 10 ⁻⁵	0.167 × 10 ⁵	<i>R</i> ₄	<i>R</i> ₈	<i>R</i> ₄ − <i>R</i> ₈	1/(<i>R</i> ₄ − <i>R</i> ₈)

The values for 1/*v*, when plotted against 1/*c*, should lie on a straight line, if this is extrapolated until it meets the 1/*v* axis at *X*, then the cytochrome oxidase activity = 1/*X*

DISCUSSION

When a reducing agent (RH)* and cytochrome *c* are added to a cytochrome oxidase preparation, two consecutive reactions occur, viz



* Since the catalyzed rate of oxidation of a reducing agent is actually measured, the latter is often termed the 'substrate' of the reaction. Such a terminology has been responsible for a confusion of thought, since the only enzyme substrate in the two reactions is cytochrome *c* which is oxidized by its specific oxidase, cytochrome oxidase The reducing agent is not the substrate of an enzyme, its function is to reduce cytochrome *c* by a non-enzymic reaction.

glutathione by determining the rate of O₂ uptake, and used as enzyme an unwashed kidney homogenate which has an appreciable endogenous respiration and probably also contains an appreciable concentration of metals which might catalyze the oxidation of glutathione and ascorbic acid More over, many of the inhibitors used, e.g. diethyldithiocarbamate, are themselves rapidly oxidized by tissue preparations in the presence of cytochrome *c*, and the degree of inhibition calculated will depend largely on the manner of correcting for this oxidation The method of making this correction is not stated by these workers There seems to be no more evidence for the existence of a glutathione dehydrogenase than for dehydrogenases activating *p*-phenylenediamine, hydroquinone, cysteine, ascorbic acid, etc

It has been known for many years that tissue preparations are able to catalyze the oxidation of *p*-phenylenediamine Keilin & Hartree (1938) showed that the addition of cytochrome *c* to the tissue preparation resulted in a greatly increased

rate of oxidation, and suggested that the oxidation in the absence of added cytochrome *c* was due to the cytochrome *c* present in the tissue preparation, it was considered that this cytochrome *c* was accessible to *p*-phenylenediamine but not to the other reducing agents, since the latter were not oxidized unless cytochrome *c* was added. Keilin & Hartree's experiments did not exclude the possibility that enzyme preparations contained an alternative pathway for the oxidation of *p*-phenylenediamine, independent of cytochrome *c* and cytochrome oxidase. Stotz *et al* (1938b) suggested that cytochrome *b* offered such a pathway, since it could be reduced by *p*-phenylenediamine and the oxidation of this compound by tissue preparations was, in contrast to that of hydroquinone, not completely inhibited by cyanide. Since Borei (1945), Laki (1938) and the writer have failed to confirm these findings, this explanation seems unlikely. Another possible catalyst for the oxidation of *p*-phenylenediamine by heart-muscle preparation is a copper-containing enzyme, like laccase. It is, however, most unlikely that heart muscle preparation contains such an enzyme, since substances such as catechol, which are readily oxidized by laccase, are not oxidized by heart-muscle preparation. The present investigation has provided strong evidence in favour of Keilin & Hartree's view, viz that *p*-phenylenediamine is oxidized in the heart-muscle preparation only by the cytochrome *c* cytochrome oxidase system. This evidence is (1) the spectroscopic observation that the cytochrome *c* in the heart-muscle preparation is much more rapidly reduced by *p*-phenylenediamine than by other reducing agents, which explains the ability of the preparation to oxidize *p*-phenylenediamine in the absence of added cytochrome *c*, (2) an abnormal heart-muscle preparation, which contained very little cytochrome *c*, did not catalyze the oxidation of *p*-phenylenediamine, unless cytochrome *c* was added, (3) a kidney preparation, which did not rapidly oxidize succinate unless cytochrome *c* was added, also required the addition of cytochrome *c* for the oxidation of *p*-phenylenediamine, (4) the activity of the cytochrome oxidase, measured by extrapolation to infinite cytochrome *c* concentration, was the same when *p*-phenylenediamine was used as the reducing agent as when ascorbic acid was used.

Stotz *et al* (1938) and Borei (1945) have concluded that cytochrome *c* forms with cytochrome oxidase a dissociable complex of the same type as postulated by Michaelis to occur between a soluble enzyme and its substrate. The only evidence on which they base this conclusion is that the effect of cytochrome *c* on the activity can be described by the Michaelis-Menten equation, which, when expressed in the modified form of Lineweaver & Burk

(1934), postulates that the inverse of the activity bears a linear relationship to the inverse of the substrate concentration. However, it does not necessarily follow from such a relationship, which has also been obtained in the present work, that a dissociable complex is formed, the only conclusion which can be drawn with validity is that the relationship velocity-substrate concentration is a rectangular hyperbola. There are, in fact, several objections to the view of Stotz *et al* and Borei, viz (1) An assumption made in deriving the Michaelis-Menten equation is that the rate of formation and dissociation of the complex is rapid compared with its decomposition into the products of the enzymic reaction. It seems unlikely that such a rapid equilibrium would be established between the insoluble cytochrome oxidase attached to the particles of the heart-muscle preparation and cytochrome *c* of molecular weight 16,500.

(2) This view does not explain the fact that the rate of O₂ uptake depends upon the particular reducing agent and on its concentration as well as on the concentration of cytochrome *c*. Since the sole function of this reducing agent is to reduce the cytochrome *c*, it follows that the rate of reduction of cytochrome *c*, as well as its oxidation, is important. The rate of reduction would be augmented by increases in the concentration both of reducing agent and of cytochrome *c*. It is also much greater when *p*-phenylenediamine is used as the reducing agent than when other reducing agents are used.

(3) According to the Michaelis-Menten equation, the effect of substrate concentration (i.e. $1/(\text{cyt } c)_i$) should be independent of the enzyme concentration. Fig 12 shows, however, that $1/(\text{cyt } c)_i$ is decreased 45% by doubling the enzyme concentration.

The effect of the cytochrome *c* concentration is therefore much more complicated than that postulated by Stotz *et al* and by Borei, even though, at any one enzyme concentration, the activities obey the Michaelis-Menten equation. But this equation, which is that of a rectangular hyperbola, merely expresses the finding that at low cytochrome *c* concentrations the rate of O₂ uptake is proportional to the cytochrome *c* concentration, while at high concentrations the rate of O₂ uptake becomes independent of the cytochrome *c* concentration and is dependent upon the activity of the cytochrome oxidase. Such a relationship would be expected, for example, if the rate of reduction of cytochrome *c* was the limiting factor at low cytochrome *c* concentrations. Indeed, at a fixed cytochrome *c* concentration the relationship between the rate of O₂ uptake and the ascorbic acid concentration is a rectangular hyperbola, and this can be explained by assuming that the rate of reduction of cytochrome *c* by ascorbic acid is the limiting factor at

low ascorbic acid concentrations. This cannot, however, be the complete explanation of the effect of cytochrome *c* on the rate of O_2 uptake, since the activity at a definite ascorbic acid concentration and infinite cytochrome *c* concentration is considerably greater than that at a definite cytochrome *c* concentration and infinite ascorbic acid concentration.

It is probable that the rate of diffusion of cytochrome *c* to and from the cytochrome oxidase is a limiting factor at low concentrations of the former. It has already been mentioned that the ascorbic acid is unable to reduce rapidly the cytochrome *c* in the heart muscle preparation, although this cytochrome *c* can be rapidly reduced by succinate and also, to a considerable degree, by *p*-phenylenediamine. It is likely then, that the cytochrome *c* in the immediate vicinity of the cytochrome oxidase can be readily oxidized by the oxidase, but cannot be easily reduced by ascorbic acid. The oxidized cytochrome *c* diffuses away and is replaced by a neighbouring cytochrome *c* molecule, which has been reduced by the ascorbic acid in solution. If the rate of this diffusion is a limiting factor, it would be understandable that the nearness of available molecules, which would depend on the cytochrome *c* concentration, would be important. This would also explain why the rate of reduction would be important, the rate of reduction must be sufficiently rapid so that the few molecules of cytochrome *c* in the immediate vicinity of the cytochrome oxidase are rapidly reduced, the turn over rate would have to be very much more rapid than that which might be calculated from the observed O_2 uptake and the concentration of cytochrome *c* in the solution as a whole. The rate of reduction of cytochrome *c* would be augmented by increases in the concentration of both reducing agent and cytochrome *c*. The importance of the reducing agent is shown in Fig. 7B, thus the apparent Michaelis constant was equal to $1.2 \times 10^{-5} M$ of added cytochrome *c* with *p*-phenylenediamine and $6.1 \times 10^{-5} M$ with ascorbic acid. The maximum activity, at infinite cytochrome *c* concentration, was, however, independent of the reducing agent. The rate of reduction of cytochrome *c* in solution by the concentrations of ascorbic acid and *p*-phenylenediamine used is very rapid, and there is no evidence that *p*-phenylenediamine reduces such cytochrome *c* more rapidly than does ascorbic acid. The greater effectiveness of low concentrations of added cytochrome *c* in the presence of *p*-phenylenediamine is probably due to the ability of the latter to penetrate more closely than ascorbic acid to the added cytochrome *c* in the immediate neighbourhood of the cytochrome oxidase. One must postulate some such difference between these two reducing agents to explain the relative rates of reduction of the endogenous cytochrome *c* of heart-muscle preparation.

When the rate of reaction is dependent, not only on the activity of the enzyme system responsible for the oxidation of the cytochrome *c*, but also on the rate of supply of the reduced cytochrome to the system, it is not difficult to see that doubling the enzyme concentration might not double the rate of O_2 uptake (Fig. 12), since the extra cytochrome oxidase 'molecules' would compete for the supply of reduced cytochrome *c*. At infinite cytochrome *c* concentration, however, the rate of oxidation is exactly doubled.

The suggestion that it is the cytochrome *c* in the immediate vicinity of the heart-muscle particles which is concerned in the reaction, not the cytochrome *c* in the bulk of the solution, explains the apparently conflicting observations that (1) the O_2 uptake depends on the ascorbic acid concentration, suggesting that the rate of reduction is a limiting factor, and (2) when ascorbic acid is added to a solution of oxidized cytochrome *c* containing a suspension of heart-muscle preparation, the cytochrome *c* is immediately reduced and remains reduced even when the solution is vigorously aerated. According to the above explanation, the cytochrome *c* in the bulk of the solution would remain reduced, although that in the immediate vicinity of the particles of the heart muscle preparation was largely in the oxidized form. When succinate is added instead of ascorbic acid, the bulk of the cytochrome *c* remains oxidized even though the succinic oxidase activity is less than that of cytochrome oxidase. This is because, in this case, the reduction of cytochrome *c* occurs not in the bulk of the solution, but on the particles of the heart muscle preparation.

The catalytic activity of the added cytochrome *c*, expressed by the value of $1/(\text{cyt } c)_i$, is affected by (1) the rate of diffusion of cytochrome *c* to the oxidase, (2) the rate of reduction of cytochrome *c* by the reducing agent, and (3) the accessibility of the reducing agent to the added cytochrome *c* in the neighbourhood of the cytochrome oxidase. Phosphate concentration has a very marked effect on $1/(\text{cyt } c)_i$, but little if any effect on $1/(\text{asc acid})_i$ (at least between 0.065 and 0.146M). This suggests that it has little influence on the rate of reduction of cytochrome *c* by ascorbic acid or on the accessibility of ascorbic acid to the cytochrome *c*, consequently its effect on $1/(\text{cyt } c)_i$ must be due to an action on the rate of diffusion of cytochrome *c* to the oxidase, caused probably by an alteration of the colloidal particles of the heart-muscle preparation.

The figures in the present paper show that the true activity (Q_{O_2}) of the cytochrome oxidase in the heart-muscle preparation, viz. 3400, is much higher than that previously reported by Keilin & Hartree (1947*a*), viz. 1420. The reasons for this discrepancy are (1) the activity measured by Keilin & Hartree's

method (5.4×10^{-5} M cytochrome c, 0.1 M-phosphate) is less than half the maximal activity, and (2) the activities calculated by Keilin & Hartree are based on the weight of the dried whole preparation, while the figures in the present paper are based on the weight of the fat-free dried preparation, which is 30 % less than the total weight of the dried preparation. It should be noted that the 1947 preparation of Keilin & Hartree* is very much more active than the 1938 preparation, which, according to Keilin & Hartree (1938), has a Q_{O_2} of 181 with *p*-phenylenediamine, and 128 with hydroquinone. This greatly increased activity is due to the introduction, in the method of preparation in 1947, of precipitation with acid at about 0°. Most of the published work on cytochrome oxidase has been performed with preparations the same as, or similar to, the 1938 Keilin & Hartree preparation.

It has been pointed out above that faulty conclusions regarding the effect of treatments of the enzyme preparation on the cytochrome oxidase may be drawn if the activity of the latter is measured at only one cytochrome c concentration. An inhibition of the rate of O_2 uptake measured at one cytochrome c concentration may be due either to an inhibition of the cytochrome oxidase or to an effect on the catalytic activity of the added cytochrome c. Which of these two factors is affected can be determined only by carrying out the experiment at different cytochrome c levels. Such a procedure may also be necessary to detect a partial inhibition of the oxidase since, at low concentrations of cytochrome c, this concentration and not the activity of the cytochrome oxidase may be the factor limiting the rate of O_2 uptake. Keilin & Hartree (1938) pointed out that this was the explanation for the small effect of carbon monoxide on the rate of oxidation of *p*-phenylenediamine in the absence of added cytochrome c.

Borei (1945) has made a comprehensive study of the action of fluoride on the cytochrome oxidase system. He found that fluoride, in fairly high concentration, inhibited the rate of O_2 uptake of various reducing agents in the presence of heart-muscle preparation and cytochrome c, but that this inhibition disappeared on extrapolation to infinite cytochrome c concentration. Borei concluded that sodium fluoride inhibits the rate of O_2 uptake by combining with the cytochrome c and so competing with the oxidase for its substrate. However, he was unable to produce any direct evidence for the formation of such a compound, since there was no alteration of the absorption spectrum of cytochrome c or of its rate of reduction by reducing agents when fluoride was added. It is clear from the above discussion that an alternative explanation

* This improved method of preparation, although not described until 1947, was used by Keilin & Hartree in 1940, when they reported a cytochrome oxidase activity of 1400

tion of the inhibiting effect of fluoride on the rate of O_2 uptake must be considered, viz that fluoride reduces the catalytic activity of cytochrome c in the system, not by combining with cytochrome c but by an effect on the enzyme preparation, in much the same manner as that of phosphate. It may be significant that, according to Borei, the inhibition due to fluoride is increased by high phosphate concentrations.* The question whether the effect of phosphate and fluoride is due to combination at a specific point in the cytochrome oxidase system, e.g. with magnesium as is the case with enolase (Warburg & Christian, 1942), or is due to a non-specific effect on the particles of the heart-muscle preparation, must await further investigation.

Most studies of enzyme kinetics have been made with a simple system consisting of the enzyme protein and its substrate of small molecular weight. The system reducing agent + cytochrome c + heart-muscle preparation provides a useful method of studying reactions between a substrate which is itself a protein and its enzyme, which is part of the macromolecular complex constituting the particles of the heart-muscle preparation. Two conclusions of general interest may be drawn from the present work: (1) that in such a system the rate of diffusion of the protein substrate to and from its enzyme may limit the rate of the reaction, and (2) that the protein substrate when attached to its enzyme may not be readily accessible to other reagents.

SUMMARY

1 It has been found that, under the conditions usually employed in determining cytochrome oxidase activity, the rate of O_2 uptake depends not only on the cytochrome oxidase activity but also on the concentrations of cytochrome c and reducing agent and on the catalytic activity of the cytochrome c.

2 A procedure suitable for studying the true activity of cytochrome oxidase is described. It is necessary to measure the rate of oxidation at different cytochrome c concentrations and extrapolate to infinite concentration.

3 Most of the reducing agents used for the estimation of cytochrome oxidase activity reduce the cytochrome c present in the heart-muscle preparation so slowly that their rate of oxidation is negligibly small, unless cytochrome c is added. *p*-Phenylenediamine, however, rapidly reduces this cytochrome c, and is rapidly oxidized by the heart-muscle preparation, this oxidation is, however, also strongly catalyzed by the addition of cytochrome c. The heart-muscle preparation does not possess a pathway for the oxidation of *p*-phenylenediamine additional to the cytochrome oxidase system.

* Borei also found the inhibiting effect of increasing the phosphate concentration in the absence of fluoride.

4 Catechol and adrenaline, which are sometimes used as reducing agents for the measurement of cytochrome oxidase activity, are not satisfactory for this purpose

5 The concentration of phosphate buffer has a very important effect on the rate of oxidation of *p* phenylenediamine and ascorbic acid by heart-muscle preparation

6 It is probable that the added cytochrome *c* when attached to its enzyme cannot be readily reduced by ascorbic acid. It is suggested that the effect of the concentrations of cytochrome *c* and reducing agent on the rate of O_2 uptake can be explained by assuming that the rate of diffusion of cytochrome *c* to and from its enzyme may limit the rate of the overall reaction

7 The effect of fluoride on the rate of O_2 uptake found by Borei is probably due not to combination of cytochrome *c* with fluoride, as suggested by Borei, but to an effect of fluoride on the particles of the enzyme preparation, causing an impairment of the catalytic activity of added cytochrome *c*

8 The true cytochrome oxidase activity of Keilin & Hartree's heart muscle preparation is expressed by a Q_{O_2} (based on a fat free dry weight) of 3400 at 38° , which is much higher than previously reported

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Studies on the Absorption of Proteins: the Amino-acid Pattern in the Portal Blood

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Although dietary protein is certainly the source of body protein, the steps by which it is transferred from the ingested food to the tissues are still incompletely known. In particular, there is considerable doubt about the form in which protein is absorbed from the gut into the portal blood stream, and the

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possible effect of the liver in altering the products of protein digestion on their way to the tissues. The tacitly accepted view is that the protein is completely broken down into free amino acids in the gut. These are absorbed into the blood stream and carried to the tissues, each of which withdraws such amino-acids from the blood as it may require for the synthesis of its own proteins. This view is based

mainly on the facts that the intestinal enzymes are able to break down proteins into amino-acids, and that after a protein meal the level of blood amino-acids rises (Van Slyke & Meyer, 1912). The rise in amino-acid concentration in the systemic blood after protein ingestion does not, however, prove that the amino acids are the actual compounds absorbed from the gut, for, if the protein were distributed in the blood stream to the tissues in intact or only partially degraded forms, the tissues, in taking up these and converting them into the required new protein, would presumably have to discard amino-acids present in excessive proportions while retaining those required for the synthesis of new protein. The unwanted amino-acids would diffuse to the blood and cause a similar rise in the total amino acid concentrations. That fragments of protein, larger than amino acids, are absorbed is suggested by a considerable amount of indirect evidence (Verzar & McDougall, 1936). We cannot, therefore, draw conclusions from changes in the blood amino-acid levels unless we know which individual amino-acids are involved, and whether the portal blood levels are higher or lower than those in systemic venous blood.

Part of the difficulty in devising experiments to decide between the possible explanations is undoubtedly technical. Owing to practical difficulties in obtaining portal blood previous workers have usually limited their studies to the changes in systemic blood. Such results necessarily provide a precarious basis, at best, for inferences regarding the composition of the portal blood. Furthermore, it has been impossible in the past to carry out even the most approximate qualitative and quantitative analyses of most of the individual amino-acids in the blood, hence observations have been limited to determinations of total free amino nitrogen which give no indication of the composition of the amino-acid mixture. Even greater difficulties are met with in attempting to determine the blood levels of nitrogen carried in the form of peptides (Christensen, Decker, Lynch, Mackenzie & Powers, 1947), indeed, no specific method for their assay in blood is as yet available.

It was decided to apply the technique of paper-partition chromatography (Consden, Gordon & Martin, 1944) to the analysis of blood obtained during protein digestion. This technique permits the recognition of nearly all the amino acids and peptides in a mixture, and, besides being of exceptional specificity, it also allows of a rough quantitative assessment of the amounts of each amino acid present. By the use of the London cannula, portal blood can be drawn for comparison with systemic blood. By combining these techniques it was hoped to obtain more definite evidence than had been possible hitherto of the mechanism of protein absorption.

Ten technically satisfactory experiments are described in which dogs were given by mouth, casein, casein hydrolysate (Amigen), ground beef, human serum albumin, and dog whole plasma protein. The latter protein was given in order to test the possibility that homologous protein might be absorbed differently from foreign (i.e. heterologous) proteins.

Fortunately an exceptional opportunity arose to check the main trends of the results by submitting some of the samples to Dr H. N. Christensen for analysis for free, and in some cases also for combined, α -amino nitrogen. His results are quoted separately in the addendum to this paper.

METHODS

General

The dog's regular kennel diet was withheld from the morning of the day before until the experiment was over. In each experiment, when possible, 100 g. of protein were given. It

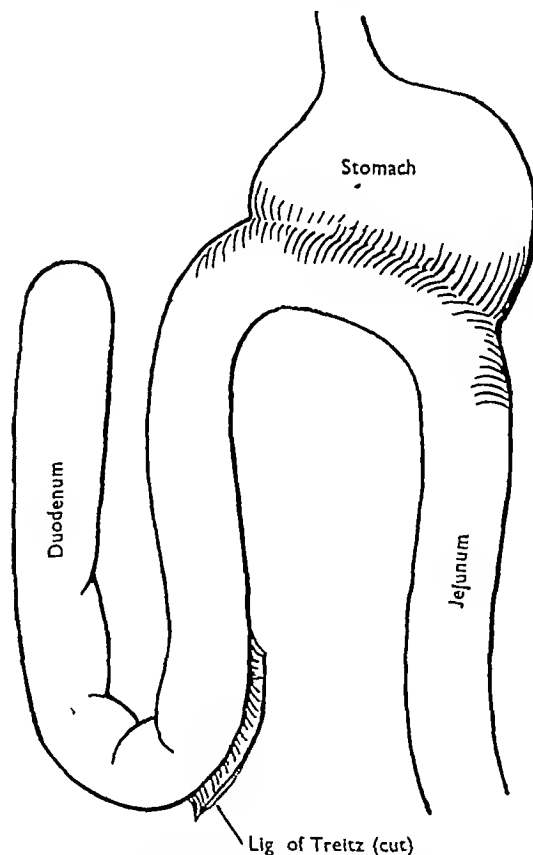


Fig 1 Diagram showing the essentials of the partial gastrectomy operation

was given as quickly as possible by stomach tube when fluid enough, by spoon, or by normal eating according to the nature of the meal. Portal and jugular blood samples were collected just before the protein administration and again 1, 2.5 and 5 hr. afterward. Each time 15 ml. were drawn and added to 0.2 ml. of 1% heparin solution. The blood was centrifuged and the plasma separated, usually within 15 min. of obtaining it. No sign of haemolysis was seen.

Operative procedures

Partial gastrectomy operation Mongrel dogs were selected that averaged 15 kg in weight. Under nembutal anaesthesia and through a left upper rectus muscle-splitting incision, the peritoneal cavity was entered. A classical Polya type of gastrectomy was performed with resection of about two thirds of the stomach, including the pylorus. The stomach was anastomosed to the first loop of the jejunum after mobilization of the duodenum with utilization of the full length of the gastric stoma, as indicated in the accompanying diagram (Fig 1). The anastomosis was performed openly and silk technique was used throughout. The wounds were all closed in layers with interrupted four zero silk sutures.

Post-operatively the dogs were allowed water *ad lib* the first day, milk and eggs until the fifth day, and then a soft cage diet of table scraps. The weight of the animals was followed carefully. Most animals lost 10–15% of their body weight and stabilized at this lower level. Blood analyses for total proteins, albumin globulin ratio, haemoglobin, red cell count, and non protein nitrogen were done at intervals. The animals appeared normal in every way. Their behaviour, appetite and activity were checked daily, and, in conjunction with the body weight, afforded a better index of the animals' condition than the blood analyses.

Insertion and operation of the London cannula London's (1935) original technique was used in the first 2 experiments. This was a two stage operation where the first stage consisted of painting the portal vein with iodine solution, and immobilizing it by sewing to the side of the adjacent part of the inferior vena cava. Two weeks later the second stage was performed, namely suturing the cannula to the painted area on the portal vein, and then wrapping it around with an elongated mass of excised free omentum. The cannula was made from a no. 16 lumbar puncture needle. A small perforated cross piece was welded on the distal end as indicated in the accompanying drawing (Fig 2). Varying lengths of cannulae could be used, depending on the size of the animal and the location of the portal vein. The cannula was brought out between the lower ribs through a stab wound, care being taken not to open the pleural reflexion. In the third and all subsequent experiments, a one stage procedure was used omitting the iodine painting and the immobilization of the portal vein, but otherwise operating as above. Only one complete failure to draw portal blood occurred in the use of this simplified technique. In addition, a correctly placed cannula, that had operated satisfactorily on one occasion, failed to draw portal blood when used for another experiment (see Exp 11). The position of the cannula was always checked carefully by post-mortem examination of the animals. In the X ray photograph (Fig 3), the general position of the cannula can be seen. The thorotrast injected into the portal vein and faintly outlining the portal radicals in the liver also serves to illustrate the method of withdrawing blood from the portal vein.

The cannula was anchored at the skin with an encircling suture and a metal cup was placed over the external stab wound. A jacket was then tied snugly around the trunk of the animal to prevent the cup from slipping, and to prevent the animal from tearing the bandages and cannula with his teeth. Liberal use of adhesive tape was useful for securely fastening the cup and jacket.

Post-operatively, the animals showed little reaction to the procedure. They were able to move with freedom in their

cages. A full diet was usually tolerated the next day though, if not, milk and eggs were supplied. The dressings were not changed until the time of the first experiment, 5 or 6 days afterwards.

It is intended to publish the technique of cannula insertion in more detail elsewhere.

Chromatographic analysis of blood

Preparation of blood samples for paper chromatography Earlier work on the application of paper chromatography to protein containing solutions had shown that although traces of protein (up to 0.5%) had little effect on the subsequent analysis, larger quantities interfered seriously, this being apparently related to the viscosity of the protein solution which acted as a mechanical barrier to the smooth soaking of the solvents along the filter paper. It was decided that ultrafiltration ought to be the best method for the removal of blood protein, and a few trials showed that this could be readily adapted to deal with the blood samples in these experiments. The following standard procedure was always used. The apparatus was that described by Greenberg & Gunther (1929). The following modification of the method of preparing the collodion sac was used. Collodion (U.S.P.) to which 4% (v/v) of ethylene glycol had been added, was poured into a 15 ml centrifuge tube which was then emptied for 30 sec while holding it at an angle of 30° to the horizontal with continual rotation. It was then clamped upside down in a vertical position for 10 min. It was again filled quickly with the collodion and emptied as before. It was now, however, drained vertically overnight. It was then held with the open end upwards, and two drops of the collodion allowed to drop down the middle to reach the tip directly. This was then dried off quickly by a light blast of air and the tube held upside down for a few hours. This extra reinforcement of the thickness of the tip was a very necessary precaution against subsequent tearing. The collodion bag now lining the centrifuge tube was ready for use. If not used at once it could be kept safely in this situation for several months without apparently affecting its final efficiency. When required, it was easy to pull it out of the tube and attach it to the apparatus. Only a very slight contamination of the product with the ethylene glycol would occur as most of the latter drained off during the drying and contraction of the collodion film. No more than 1 in 20 of these sacs would burst or develop leaks during the ultrafiltration. They usually burst if the pressure was raised to 250–300 mm of Hg. These membranes allowed inulin (mol wt c 5000) to filter through quantitatively. Cysteine (mol wt c 13,000) was, however, completely retained.

Ultrafiltration procedure The ultrafiltration of the heparinized plasma was carried out at a pressure of 150–200 mm of Hg, the whole apparatus being immersed in an ice bath. About half of the volume of plasma taken would be collected as crystal clear protein free filtrate in 8 hr. Very little more came through in a longer time, although, for reasons of convenience, the apparatus was usually run overnight. The ultrafiltrates were kept at 4° for the few days while they were awaiting chromatographic analysis. This scale of operation was chosen for convenience of manipulation, to eliminate to a negligible degree the possible loss of substances by absorption on to the collodion, and because other analytical methods requiring larger volumes were envisaged. As far as the paper chromatography was

concerned the whole analysis could have been carried out on less than one tenth of the volumes taken above

Paper chromatography method Volume of ultrafiltrate taken The standard volume of 125 μ l was taken throughout. As this was too large a volume to apply directly to the paper it was always evaporated almost to dryness on a watch glass by blowing air over it at room temp by means of an electric fan. The residue was then transferred to the paper followed by 3 washings of the watch glass. This volume was chosen as, by its use, a normal blood with NH_2N conc of 4 mg/100 ml would produce on the chromatogram up to 12 weak spots from the amino acids present in greatest amount. An increase of 50% in NH_2N conc would be easily picked up by the appearance of further amino acids, as well as by a strengthening of those seen before. The ninhydrin colour reaction as carried out on the paper becomes less sensitive at much higher concentrations of amino acids and an increase of 50% may, therefore, not be observed in such a case. To compare high concentrations less ultrafiltrate would have to be taken to bring the spots down to the more sensitive range.

Procedure Two dimensional chromatograms were used almost exclusively. Phenol was used as the first solvent, in the presence of a trace of NH_3 vapour, and collidine lutidine mixture in the presence of a trace of diethylamine as the second. Further details are given by Dent (1948).

Distortions on the chromatogram The final positions occupied by aspartic and glutamic acids on the chromatograms were always abnormal and the spots in question partly distorted into streaks. This phenomenon was due to the presence of three substances which appeared as large yellow spots and which took precedence in the occupation of certain areas of the paper with the result that the amino acids were left to surround the margins of these areas (Figs 4-9). Glutamine was sometimes affected in a similar manner. The interference was always similar in character. Once, therefore, the new positions of the amino acids had been determined, it was still possible to carry out satisfactory identifications although the rough quantitative estimations were somewhat jeopardized.

Recent work by Westall (1948) suggests that two of the yellow spots (A and C in Fig 7) are probably due to inorganic acid and to sodium phenoxide, respectively, formed by the separation on the chromatograms of the ions of inorganic salts. The present writers have recently applied the dc salting device of Consden, Gordon & Martin (1947) to plasma ultrafiltrates and have obtained undistorted chromatograms from as much as 625 μ l. This represents a considerable advance on the technique as used in the present work.

Identification of amino-acids This was carried out by the usual methods (Dent, 1948), i.e. careful position matching with markers followed by confirmation that the substances were stable to hydrolysis, and, except for methionine and cystine which are oxidized, were also stable to treatment with H_2O_2 . Once one sample of fasting dog blood had been checked in this way the identification thus obtained was assumed to apply to the spots given by fasting blood from other dogs. In the blood drawn after the proteins had been fed only the serial sample which contained the highest NH_2N concentration was checked, and this only by prior hydrolysis and H_2O_2 treatment. It was not considered necessary to recheck all the amino acids with markers in such cases. Leucine and isoleucine, which overlap on the chromatograms, were always considered together under the

name 'leucines'. It should be emphasized that the substance, moving to a position close to and just below alanine in the chromatograms as represented in this paper, was always shown to be glutamine and not the peptide 'under-alanine' (Dent, 1947), which also moves to the same place.

Method of recording strengths of spots The colour strength of each spot was recorded against an arbitrary colour chart divided into 10 parts. A strength of 1 represented a very weak purple colour, 10 an intense purple. When the colour could not be seen directly, but was just visible when the paper was viewed by transmitted light, it was called <1. Colour strength of greater than 10 (recorded as >10) or much greater than 10 (≥ 10) were well into the range where the colour reaction was much less quantitative. The strengths of the yellow ninhydrin colours such as are given by proline were assessed by plus signs.

Detection of peptides For the purpose of this paper a substance is called a 'peptide' if it moves to a characteristic position on the chromatogram not corresponding to a known substance, if it gives a typical ninhydrin colour reaction, and if it is destroyed on acid hydrolysis (see below) with simultaneous liberation of free amino acids. Isolation and identification of peptides was considered to be beyond the scope of this paper.

Whether or not peptides are detected when they are present in only small amount depends entirely on whether they are present as only a few distinct members, say 3 or 4 dipeptides, or as a very large number of di-, tri-, and polypeptides. In the latter case a larger amount of peptide N could be undetected if it were widely distributed about the paper, each peptide being present in too small a quantity to give a visible ninhydrin colour. The only way to detect this would be by observing an otherwise unaccountable increase in free amino acids after acid hydrolysis. Such an increase could also occur, however, from other sources, e.g. from acyl derivatives of amino acids such as hippuric acid. There is some indirect evidence at least in the cases of aspartic and glutamic acids (Dent, 1947) for the existence of further members of this series. The finding of such an increase in amino acids on hydrolysis is more safely described, therefore, as being due to 'bound amino acids'. A hydrolysis was performed on at least one of the ultrafiltrates of the blood samples from each experiment whether or not more direct evidence of the presence of peptides had been obtained. It was estimated that bound amino acids, if involving all the amino acids in similar relative proportions to their occurrence in the free state, could be present containing up to 20% of the total NH_2N without being detectable on the chromatograms. On the other hand, if the bound amino acids concerned only one or two members then the method would be far more sensitive. These opinions are based on the results of experiments with synthetic peptides and with proteins partly hydrolyzed by enzymes, acids and alkalis.

Acid hydrolysis of samples The sample (125 μ l) was mixed with an equal volume of concentrated HCl, sealed in a capillary tube and immersed in boiling water for 24 hr. It was then transferred to a watch glass, evaporated to dryness at 60°, and the residue washed on to the filter paper, with about 30 μ l of water, applied in several lots.

Brief summary of each dog experiment

Exp 1 (dog 461) Casein, 28 January 1947, wt 16 kg, first stage of cannula insertion, 10 January 1947, second stage 24 January 1947. Casein (100 g) damped with about

200 ml of water was fed by spoon. Blood samples were apparently collected satisfactorily. The dog was killed after the 5 hr period. The stomach was found to be full of casein, and also contained some bandages, presumably from the dressings. It was thought that the latter might have produced some obstruction to the further passage of the casein. In view of the lack of absorption of the casein the blood samples were rejected.

Examination of the cannula insertion showed that the inferior vena cava contained a series of recent punctures while the adjacent portal vein was intact. Clearly the 'portal' blood samples taken in this experiment were really of systemic origin.

Exp 2 (dog 46 10) Casein, 17 February 1947, wt 11 kg, first stage of operation 28 January 1947, second stage 12 February 1947. Casein (100 g) fed by spoon. The blood samples were drawn without difficulty. The dog was killed after the 5 hr period. The stomach was found to contain 37 g of apparently unchanged casein. It is possible, therefore, that the bandages were not the cause of the delay in stomach emptying found in *Exp 1*. It was as a result of this that the decision to do the prior partial gastrectomy operation was taken. The cannula had been working very well in this experiment and only the portal vein had been pierced by the sampling needle.

Exp 3 (dog 46 32) Dog plasma, 24 March 1947, wt 9 kg, partial gastrectomy operation 3 June 1946, cannula inserted 18 March 1947 (From now on this was done by the one stage method, see p 320). Freeze dried (lyophilized) dog plasma protein (100 g) made up to 400 ml with water was given by stomach tube. Considerable gastro intestinal disturbance resulted. The dog vomited a large amount 10 min afterwards. This was given back to the dog. A further vomit 40 min later was also given back by tube. A small amount of diarrhoea occurred 15 hr later. This was rejected. It was estimated that not more than 25 g in all of the original protein could have been lost during these incidents. The blood samples were taken very satisfactorily. It was, therefore, decided to use the same dog for the next experiment, and to repeat later the protein feeding with dialyzed plasma, as the gastro intestinal upset might have been due to the high salt content of the concentrated plasma.

Exp 4 (dog 46 32) Casein 28 March 1947, same dog as *exp 3*, wt now 9.6 kg. Casein (100 g) fed by spoon. Blood samples taken satisfactorily, although on one occasion when perhaps the needle was inserted a little farther than usual, pure bile was withdrawn in the syringe. Urine passed at 1.5 hr was collected. At 4.5 hr the dog vomited 250 ml of bile stained liquid, containing much mucus and a small amount of casein. The dog was killed after the 5 hr period. The cannula was found to have been working satisfactorily and only portal blood had been drawn through it. The gall bladder and bile duct were distended, presumably as the result of a stricture produced by adhesions around the cannula. There was therefore some degree of biliary obstruction, and it was easy to see how the sample of bile had been accidentally withdrawn. The stomach and intestines were empty and normal in appearance. The liver also appeared normal, sections were taken and found to be normal microscopically. Blood analyses showed icterus index, 9 units, bilirubin, 0.13 mg/100 ml (direct) and 0.42 mg/100 ml (total).

Exp 5 (dog 46 178) Casein, 16 June 1947, wt 15.5 kg, partial gastrectomy operation 13 January 1947, cannula inserted 11 June 1947. Casein (100 g) fed by spoon. Blood

samples taken satisfactorily. Dog was well throughout the experiment. After the 5 hr period had been passed the dog was X rayed while thorotrast was being injected through the cannula. The picture (Fig 3) confirmed that the needle was inserted into the portal vein. The dog was then killed. The cannula was seen to be well placed and the inferior vena cava unpunctured. The stomach and intestines were empty except for a few small particles of undigested casein in the upper part of the small intestine.

Exp 6 (dog 47 11) Dog plasma, 28 June 1947, wt 13.5 kg, partial gastrectomy 20 January 1947, cannula inserted 23 June 1947. Dialyzed and freeze dried dog plasma protein (100 g) was made up to 400 ml with water and given by stomach tube. Blood samples taken satisfactorily. Portal and jugular blood was also taken at 8 hr in this experiment. This animal did not vomit, but 15-22.5 hr after it had been fed by tube it had some considerable diarrhoea. The dog excreted material which closely resembled the protein solution originally fed. Fortunately, however, the dog avidly ate up at once everything he had passed, so that only a few g protein were lost. The dog was kept for a further experiment.

Exp 7 (dog 47-11) Amigen, 3 July 1947, same dog as in *Exp 6*, well since then. Hydrolyzed casein (Amigen, 100 g) stirred with 300 g of warm water was given by stomach tube. The dog appeared well throughout the experiment. The blood samples were taken satisfactorily and, in addition, a sample of urine passed at 4 hr. The dog was killed after the 5 hr samples were taken. At post mortem the correct position of the cannula was confirmed. The gut was apparently empty.

Exp 8 (dog 47 60) Dog plasma, 23 July 1947, wt 10 kg, partial gastrectomy 18 February 1947, cannula inserted 17 July 1947. The dog was given, by stomach tube in two equal portions 15 min apart, 800 ml of heparinized dog plasma. This had been drawn from donor dogs two days previously and kept frozen solid. It contained at least 50 g of dry plasma protein. From the 0.5 hr period after feeding to 2 hr after, this dog vomited intermittently. The vomit was collected each time and given back by tube, at the most about 50 ml of the original 800 ml being lost. There was no diarrhoea. The blood samples were taken satisfactorily. After the last sample the dog was killed. At post mortem the cannula was found to be correctly placed. The stomach and small intestine were empty, but the large intestine contained some semi liquid faecal material. The entire gut from stomach to rectum was carefully squeezed out. The material measured 65 ml, appeared to be only faeces and contained 1.6 g of total N, corresponding to about 10.0 g of total protein ($N \times 6.25$).

Exp 9 (dog 47 48) Human serum albumin, 1 August 1947, wt 11 kg, partial gastrectomy 24 February 1947, cannula inserted 26 July 1947. The dog was given by stomach tube 50 g human albumin (200 ml of 25% solution) at 0 hr and another 50 g 0.5 hr later. The albumin was described as 'concentrated salt poor human serum albumin' (Squibb and Sons, New York). It contained 1% of DL acetyltryptophan as stabilizer. The dog tolerated this dosage very well, appearing lively and well throughout, in marked contrast to the gastro intestinal upset in the experiments in which dog plasma was fed. Blood samples were taken satisfactorily. Urine was passed at the start of the experiment, and a sample collected when the bladder was emptied at 5 hr. The dog was kept for further experiments.

Exp 10 (dog 47 48) Denatured dog plasma, 6 August 1947, same dog as in *Exp 9* Heat-denatured plasma protein (80 g) obtained from donor dogs was fed by spoon quite satisfactorily. About 0.5 hr later the dog vomited what appeared to be the entire amount of protein. Half of this was fed back to the dog, but was again vomited shortly after. In view of the apparently complete intolerance to this substance the experiment was discontinued.

Exp 11 (dog 47 48) Ground beef (Hamburger), 7 August 1947, same dog as in *Exp 10*, wt 10.5 kg. Raw ground beef (500 g, c. 105 g of protein) with the fat stripped off as much as possible before mincing, was fed 0.5 hr later the dog vomited some apparently unchanged meat mixed with mucus. Some of this was eaten again quickly. The remainder weighed 192 g and was rejected. Therefore at least 308 g containing about 65 g of protein were retained. The blood samples were taken satisfactorily. After the 5 hr period the dog was killed. The post mortem showed that the end of the cannula had slipped from the portal vein. The portal vein showed some brown pigmented spots where it had presumably been punctured the week before (*Exp 9*). On the other hand, the inferior vena cava showed recent red staining undoubtedly over the sites of recent needle pricks. The gut was found to be fairly full of dark smelly liquid. This was considered to be a normal residue after eating meat.

Exp 12 (dog 47 67) Ground beef, 14 August 1947, wt 18 kg, partial gastrectomy 8 April 1947, cannula inserted 8 August 1947. Raw ground beef (500 g) as fat free as possible was fed. The blood samples were taken satisfactorily. The dog was lively and well throughout the experiment. It was saved for further trials.

Exp 13 (dog 47 67) Human serum albumin, 16 August 1947, same dog as in *Exp 12*. Dog was fed 400 ml of 25% human albumin (same material as in *Exp 9*) by stomach tube. This was well tolerated, the dog appearing lively throughout. Blood samples taken satisfactorily. Urine was collected when the bladder was emptied at the beginning of the experiment, and again after 4 hr.

The dog was saved for a further experiment on 20 August 1947 (not reported here) in which glutamic acid was fed. After this experiment the dog was killed. A post mortem then gave unequivocal evidence that the cannula had been correctly placed.

RESULTS

Amino acid analyses by one dimensional chromatograms This method was used as a rough sorting test to detect potentially interesting fluids. When one was found, e.g. the urine after feeding human albumin, the fluid was tested further on two-dimensional chromatograms (Tables 2 and 3) so there is no need here to state the result of the rough test. In the urines obtained after feeding casein (*Exps 2 and 4*) there were no abnormal amino-acids detectable in the one dimensional chromatograms so no further tests were done. Unfortunately, no urine was tested after the feeding of dog plasma, although it could hardly be expected to have been abnormal, owing to the normal blood levels of amino acids.

Amino acid analyses using two-dimensional chromatograms These are summarized in Tables 1-3. A series of original chromatograms is much easier to interpret than the tables. A few chromatograms are, therefore, reproduced photographically or as diagrams in Figs 4-15. Tryptophan and hydroxyproline, although easily detectable if present in sufficient quantity, have not been seen in any of the blood samples. They are not, therefore, mentioned in the tables. Asparagine is also omitted although it has been found once (see Fig 11). There is considerable doubt as to the identity of citrulline. The spot given in this position by normal blood has the characteristic reddish purple colour of citrulline, although there is not enough present to confirm the identity by the spot test with *p*-dimethylamino-benzaldehyde (Dent, 1948). It is possible, therefore, that the spot could have been due to β alanine, which moves to a similar position, although it usually gives a ninhydrin colour having a bluer shade. In one hydrolyzed urine (*Exp 13*, 0-4 hr) the spot was certainly due chiefly to β -alanine as there was sufficient quantity present to do the chemical test. Reasons mentioned in the Discussion suggest that the increase in strength of the spot so often found after acid hydrolysis might always be due to β alanine.

The order of the amino-acids in Tables 1-3 is roughly that in which they are spread out on the paper. This order soon becomes familiar to workers using the chromatograms. The numbers under each substance refer to the colour strength of the ninhydrin reactions on the arbitrary scale of 10. They are of relative value only. The strength of the aspartic acid spot was often difficult to judge owing to the distortion always present.

Analyses for α -amino nitrogen by chemical methods The following results were obtained by Dr H. N. Christensen with the methods described in the addendum to this paper.

Dog (Exp 4) Analysis of ultrafiltrate of vomit ejected 4.5 hr after giving the 100 g of casein: total α amino nitrogen, 440 mg/100 ml, free α -amino nitrogen, 69 mg/100 ml.

Dog (Exp 13) Analysis of urine passed just before human albumin was given, in mg α amino nitrogen/100 ml: total 63.1, free 16.0, bound 47.1. The next urine specimen was passed 4 hr after the experiment had begun and contained total 41.7, free 34.2, bound 7.5.

Electrophoretic analysis of portal plasma after feeding human-serum albumin Human-serum albumin injected intravenously into dogs can be readily detected in the plasma by electrophoretic means many hours after the injection (McKee & Alling, 1946). Dr Alling kindly examined in this way the portal plasma 2.5 hr after feeding human-serum albumin in *Exp 13*. No peak corresponding to

the human albumin was seen and the portal plasma at 2.5 hr matched in every respect the fasting sample at 0 hr. By this method, it was

on plasma protein content the specific gravity of the specimens of portal and jugular plasma was determined by the copper sulphate method

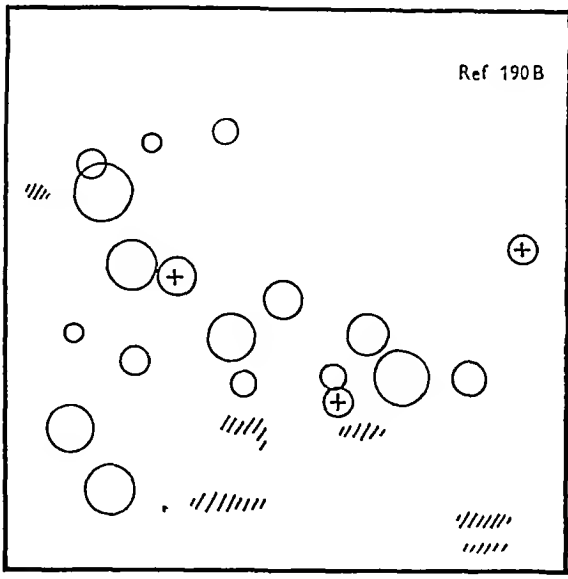


Fig 10

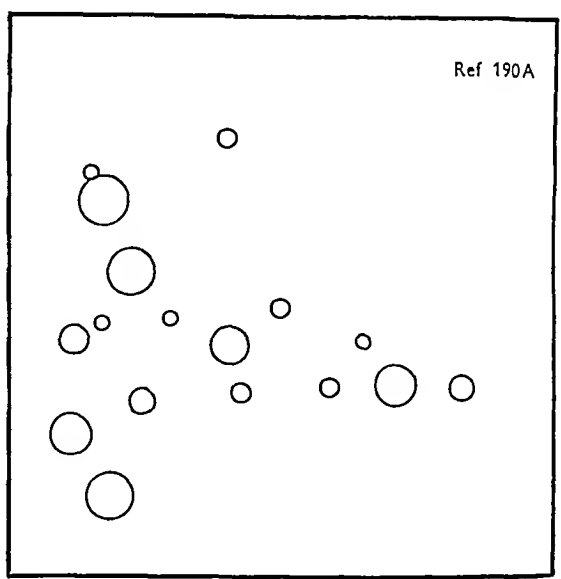


Fig 12

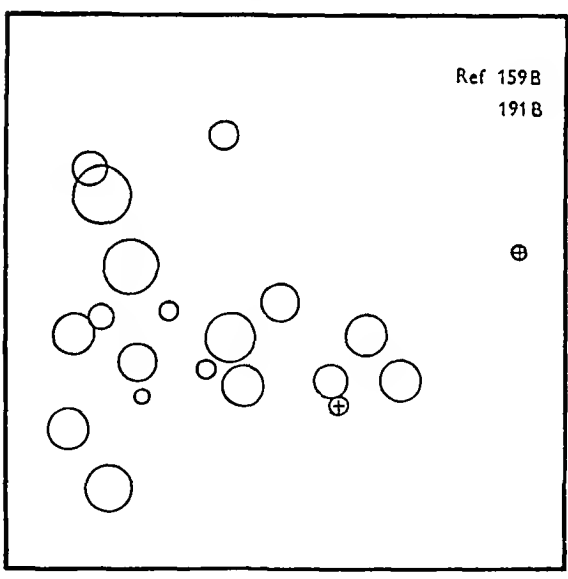


Fig 11

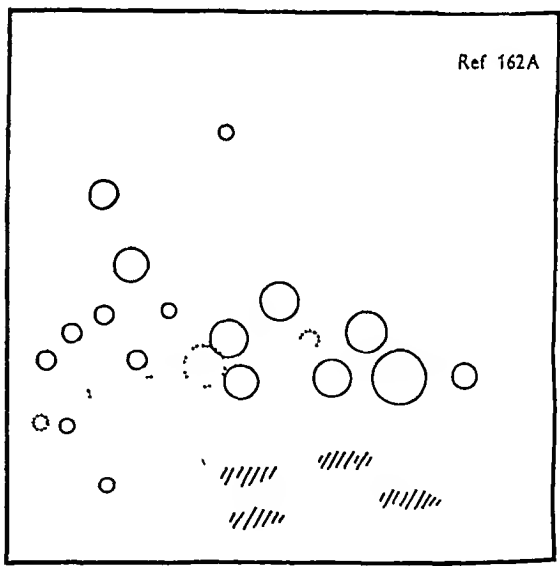


Fig 13

Figs 10-13 Diagrams of chromatograms obtained during the feeding of casein hydrolysate (Amigen). In these figures, the size of the spots has been drawn to represent the strength of the ninhydrin colour. The identifications can be made from Fig 7. Aspartic and glutamic acids are, however, shown in these diagrams in their true positions, not in the distorted positions as in Fig 7. The three further spots in Fig 10 (see also Fig 11) marked in the centre with crosses are, from right to left, cysteic acid (from cystine), asparagine, and methionine sulphone (from methionine). The spots outlined with a dotted line or shown by shading represent 'peptides'. The materials run on each chromatogram were: Fig 10, 300 μ g of Amigen; Fig 11, 125 μ l of the 1 hr portal blood ultrafiltrate; Fig 12, 125 μ l of the 1 hr jugular blood filtrate; Fig 13, 25 μ l of the urine secreted in the first 4 hr of the experiment.

estimated that at least 10 g of circulating human-serum albumin could be detected. Hence of the 100 g fed little if any could have been absorbed intact.

Specific gravity of portal plasma. As a rough check

(Philips, Van Slyke, Dole, Emerson, Hamilton & Archibald, 1945). No significant change in specific gravity was found on any of the specimens taken during Exps 5 (casein), 6 (dog plasma), and 9 (human serum albumin).

Table 1. Levels of amino acids in body fluids

(Figures indicate arbitrary units, see p 321)

Sample analyzed	Exp 2 100 g casein (dry wt) fed										Exp 3 100 g dog plasma protein (dry wt) fed About 25 g subse- quently vomited										Exp 4 100 g casein (dry wt) fed vomited at 4.5 hr										About 37 g																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
	Plasma filtrate					Plasma filtrate (hydrolyzed)					Plasma filtrate (hydrolyzed)					Plasma filtrate (hydrolyzed)					Plasma filtrate (hydrolyzed)					Plasma filtrate (hydrolyzed)					Vomit filtrate																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
	P	P	J	P	P	P	J	J	J	J	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P

* Large numbers of peptides also seen

+ All the peptides have now disappeared

Methionine is seen partly overlapping leucine There is also an unidentified substance 'fast arginine' (see Dent, 1947)

Table 2 *Levels of amino-acids in body fluids*

(Figures indicate arbitrary units, see p 321)

	Exp 5 100 g casein (dry wt) fed					Exp 6 100 g dog plasma protein (dry wt) fed					
Sample analyzed	Plasma filtrate	Plasma filtrate	Plasma filtrate (hydro-lyzed)	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate (hydro-lyzed)	Plasma filtrate	Plasma filtrate
Time of sample (hr)	0	1	1	2.5	5	0	1	2.5	2.5	5	8
Portal or jugular	P	P	P	P	P	P	P	P	P	P	P
Cysteic acid (from cystine)		<1									
Aspartic acid											
Glutamic acid	2	6	8	6	2	1	1	<1	6	<1	<1
Serine	1	6	3	6	3	1	1		<1	<1	<1
Taurine	3	3	1	2	<1	<1	<1				
Glycine	1	2	3	2	<1	<1	<1		<1	<1	<1
Threonine		4	4	4	1						
Alanine	6	8	9	8	6	3	3	1	4	1	1
Glutamine	4	6		7	4	3	3	2		2	2
Tyrosine		5	4	5	2						
Citrulline and/or β alanine	1	1	2	1					1		
Histidine			4								
α -Amino-n-butyric acid	<1	2	<1	2	2	<1	<1	<1	1	<1	<1
Methionine sulphone (from methionine)		1									
Valine	6	9	8	8	7	5	5	5	5	4	2
Methionine sulfoxide		3	4	3	2	?	?				
γ -Aminobutyric acid		<1									
Lysine	1	7	7	6	4		<1		1		
Phenylalanine		3	3	2							
Leucines	1	9	9	8	7	3	2	2	3	1	1
'Fast aminobutyric acid'	<1	1		<1	<1	<1	<1	<1	1	<1	<1
Proline	<+	++	++	++	+						
Arginine	2	6	7	5	3	<1	<1		2		1

	Exp 7 100 g casein hydrolysate (Amigen) (dry wt) fed							Exp 8 50 g dog plasma protein (dry wt) fed					
Sample analyzed	Plasma filtrate	Plasma filtrate *	Plasma filtrate (hydro-lyzed) †	Plasma filtrate	Plasma filtrate	Plasma filtrate	Urine ‡	Urine (hydro-lyzed) §	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate (hydro-lyzed)	Plasma filtrate
Time of sample (hr)	0	1	1	1	2.5	5	0-4	0-4	0	1.5	3	3	5.5
Portal or jugular	P	P	P	J	P	P			P	P	P	P	P
Cysteic acid (from cystine)		<1											
Aspartic acid			?	2			2	4					
Glutamic acid	1	6	10	6	5	3	9	≥ 10	<1	<1	2	6	2
Serine	<1	6	6	<1	5	1	6	6	<1	<1	1	<1	<1
Taurine	1	<1	<1						<1	<1	<1	<1	<1
Glycine	1	4	5	1	4	<1	5	7	<1	<1	<1	<1	<1
Threonine		5	6	1	6	4	5	6		<1	1	1	<1
Alanine	6	8	10	5	7	4	5	8	2	3	4	3	4
Glutamine	4	6		1	3	2	4		2	2	2		2
Tyrosine		3	3	1	3	1	<1	1			<1	<1	<1
Citrulline and/or β alanine	<1	1	4		1	1	6	4			<1	<1	
Histidine			3		<1			1					
α -Amino-n butyric acid	1	1	1	<1	1	1		<1			<1	<1	<1
Methionine sulphone (from methionine)													
Valine	4	9	10	7	10	8	4	4	3	3	6	5	6
Methionine sulfoxide		5	4	2	5	4	1	2			<1	<1	<1
γ Aminobutyric acid	1	<1						1	<1	<1	<1	<1	<1
Lysine	<1	7	9	7	8	5	1	<1	<1	<1	2	2	2
Phenylalanine		4	4	<1	3	2		<1			<1	<1	<1
Leucines	3	10	10	8	10	7	3	4	2	2	5	5	5
'Fast aminobutyric acid'	1	2		<1	<1	<1				<1	<1	<1	<1
Proline	<+	++	++	++	++	+	+	+	1	1	<+	2	<+
Arginine	1	6	8	6	6	3	<1	<1			2	2	2

* Trace asparagine seen.

† Two new spots also seen

‡ Many peptides also seen

§ One new spot also seen.

Table 3 Levels of amino acids in body fluids

(Figures indicate arbitrary units, see p 321)

Sample analyzed Time of sample (hr) Portal or jugular	Exps 11 and 12 105 g ground beef fed (dry wt)										Exp 13 100 g human serum albumin (dry wt) fed									
	Exp 11 (35 g of protein vomited)					Exp 12					Plasma filtrate									
	Urino (hydrolyzed)					Plasma filtrate (hydrolyzed)					Plasma filtrate (hydrolyzed)									
	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Plasma filtrate	Urine (hydrolyzed)§
Time of sample (hr)	0	1	2	5	0-5	5	5	5	5	5	0	1	1	1	1	1	1	1	1	Urine (hydrolyzed)§
Portal or jugular	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	Urine (hydrolyzed)§
Cysteic acid (from cystine)																				
Aspartic acid	1	1	1	1	2	3	3	3	3	3	1	1	1	1	1	1	1	1	1	2
Glutamic acid	2	<1	2	5	<1	>10	>10	>10	>10	>10	2	4	6	6	4	1	4	8	9	6
Serine																				
Taurine																				
Glycine																				
Threonine	<1	<1	3	<1	8	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	6
Alanine	3	1	5	7	4	6	6	6	6	6	2	1	1	1	1	1	1	1	1	>10
Glutamine	3	2	2	1	8	8	8	8	8	8	3	7	7	7	5	3	5	8	9	<1
Tyrosine																				
Citrulline and/or β alanine																				
Histidine																				
α Amino α butyric acid																				
Methionine sulphone (from methionine)																				
Valine	4	1	9	10	7	<1	5	9	4	6	7	9	9	7	2	6	10	10	9	8
Methionine sulphoxide																				
γ-Aminobutyric acid	<1	<1	?	1	?	2	2	2	2	?	<1	2	2	2			1	1	1	?
Lysine	2	3	6	9	5	6	7	6	6	2	4	6	8	6	3	6	<1	10	7	?
Phenylalanine																				
Leucine	4	5	8	10	6	1	1	<1	<1	<1	5	8	9	<1	2	1	4	5	1	>10
'Fast aminobutyric acid'	<1	<1	1	1	<1	7	7	8	<1	<1	<1	<1	9	7	2	7	10	10	8	10
Proline																				
Arginine	3	4	5	7	3	3	3	5	3	4	6	6	6	6	2	5	8	8	6	9

* Many peptides also seen

§ Many peptides and unknowns

† Inferior vena cava

‡ Many unknowns also, peptides have disappeared

† Methylhistidine and unknown amino-acids also

Summary of results given in Tables 1-3

Casein Ingestion of this protein caused large rises in portal and jugular concentrations of amino-acids. The highest concentration of amino acids was obtained in Exp 5. With the exception of glutamic acid, the rise in individual amino acids was consistent with what would be expected from the complete absorption of a casein hydrolysate (Figs 8 and 9). It is remarkable that glutamic acid which occurs in casein to the extent of over 20% should not increase more in the portal blood during the digestion. As the two samples in Figs 8 and 9 were matched as to total amounts of amino nitrogen, clearly the excess of glutamic acid in Fig 9 has to be associated with a weakening of the spots due to the other amino acids.

The vomit obtained in Exp 4, 4 hr after feeding the casein, showed a very large concentration of amino-acids and of peptides. The free amino nitrogen concentration could be roughly estimated as being about 20 times that of normal blood.

Casein hydrolysate (Amigen) As expected, ingestion of this was followed by large rises in portal amino acids and only slightly smaller rises in the jugular blood. No appreciable synthesis of protein or of peptides by the intestinal mucous membrane could have occurred. The rate of absorption was only slightly faster than in the case of unhydrolyzed casein. The passage of this substance through the body can be seen best in the diagrams in Figs 10-13. The original Amigen contains 'peptides' as can be seen more readily than on Fig 10 by running chromatograms on larger amounts of Amigen or on concentrated fractions. The 'peptides' tend to concentrate in the urine, relative to the amounts of free amino acids excreted (see also Christensen, Lynch & Powers, 1946). The figures also show well the invariable action of the kidneys in retaining preferentially the essential amino acids and not so well the unessential. For instance, leucine is present in relatively smaller quantity in the urine than in the blood, glycine in relatively larger amounts.

Ground beef (Hamburger) Ingestion of this also resulted in large increases in portal amino-acids. The peak of digestion at 2.5 hr, however, corresponded to a lower concentration of amino acids in the portal blood than was found after feeding equivalent amounts of any of the other proteins. There was evidence of a little bound amino nitrogen in the blood collected at this time. The peculiar composition of the 1 hr specimen is also worthy of note. There was a rise in the concentration of some amino acids, accompanied by a fall in the concentration of others, thus making an estimate of total amino nitrogen change much more difficult. If a change did occur in total amino nitrogen it could not have been appreciable.

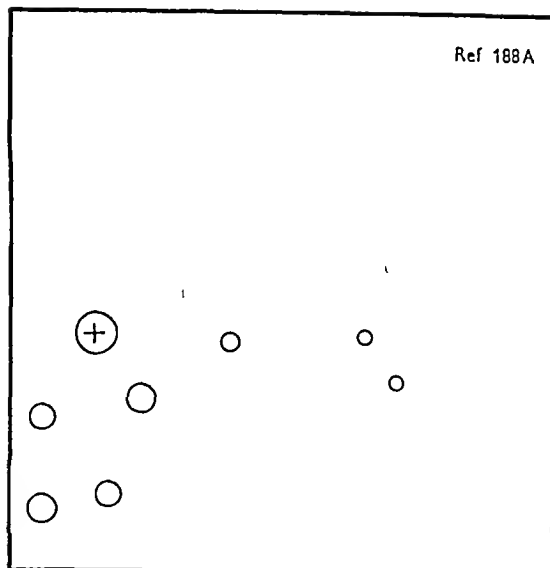


Fig 14

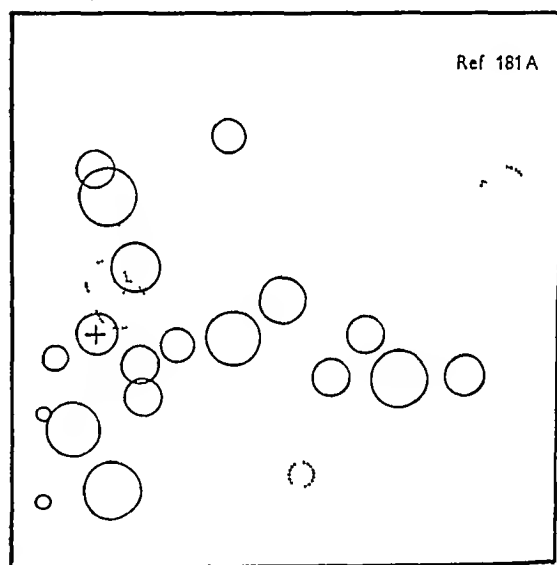


Fig 15

Figs 14 and 15 Diagrams of the chromatograms from the urines of Exp 13 (human serum albumin feeding). The same conventions have been used as in Figs 10-13. The three small spots above each other along the left-hand margin of Fig 15, and the two corresponding ones of Fig 14, represent unknown amino acids, the middle one is in the position occupied by 'fast arginine', a basic amino acid already found in other sources (Dent, 1948). The spot marked with a cross is believed to be due to methylhistidine. The material analyzed on each chromatogram was Fig 14, 25 μ l of the urine passed just before the experiment was begun, Fig 15, 25 μ l of the urine secreted in the first 4 hr of the experiment. Note the gross amino aciduria and peptiduria in Fig 15.

Human serum albumin The rise in amino acid concentrations in the portal blood in Exp 13 was the highest of any of these experiments (Fig 6). Even at this concentration of digestion products, no

peptides were detected. There was, however, some bound amino nitrogen. The urine showed spectacular changes (Figs 14 and 15). There was a gross amino-aciduria, at least four definite 'peptides' were present and the excretion of glutamic acid was phenomenally high. There were also some unknown amino acids and on hydrolysis a large rise in the content of histidine and of β -alanine occurred. The large amount of β -alanine, which gave no colour reaction with *p*-dimethylaminobenzaldehyde, allowed of a definite distinction from citrulline. This extraordinary picture was exactly reproduced qualitatively, although in slightly less concentration, in the other experiment (Exp 9) in which human serum albumin was fed more slowly to a different dog.

Dog whole-plasma protein. Ingestion of this protein was not followed by any appreciable changes in the portal blood levels of amino-acids. The contrast between this result and those obtained with other proteins is definite and unambiguous. The best experiment technically was Exp 6 because all the 100 g of protein fed was retained, and the portal blood was followed for 8 hr altogether. There seemed to be a slight but definite fall in plasma amino acid concentration in the middle specimens. There was a slight rise in amino-acid concentration towards the end of Exp 8. This was much less than in any of the experiments feeding other proteins. It may, however, be that this does represent slight digestion of the protein. In this experiment the gut was confirmed to be almost empty 5 hr after the feeding, so there is no question of the protein having remained in the gut unchanged and unabsorbed.

DISCUSSION

The results obtained here show that large rises in the concentrations of many amino acids occur in the portal blood of the partially gastrectomized dog after ingestion of casein, casein hydrolysate, ground beef and human serum albumin. In marked contrast to this, however, in the three experiments in which dog whole-plasma protein was given, only relatively insignificant changes were found in the portal blood, although in one dog it was shown that the protein had disappeared from the gut at the end of the experiment.

In the group of experiments in which the heterologous proteins were given to the dogs, it has been shown that the blood amino acids and other ninhydrin-reacting substances fall into three groups, those which rise and fall markedly and at about the same rates in their blood concentrations after the protein meal, those which vary less regularly tending if anything to rise slightly throughout the 5 hr period, and those which are present in only

small quantities and do not change appreciably during the experiment. The first group comprises the amino-acids commonly found in protein hydrolysates. This group is not completely accounted for, since hydroxyproline and tryptophan, which, present in small amounts in the proteins fed, would be expected to occur only in low concentrations, were never detected, and since methionine and cystine were not looked for systematically. Methionine sulphoxide, although not thought to occur in proteins, was detected and appeared also to belong to this group (Exps 5 and 7). The second group comprises α - and γ -aminobutyric acids and perhaps glutamine. The third group comprises taurine, citrulline (and/or β -alanine) and the unidentified amino acid 'fast aminobutyric acid'.

The amino acids belonging to the first group are interpreted to represent the most important, possibly the only, compounds by which the original protein is transferred from the gut into the portal blood prior to its delivery to the liver and tissues. This view is based partly on the fact that these amino-acids are those which are liberated by hydrolysis of the protein in question. In one experiment (Exp 5) in which casein was fed, it was further possible to show chromatographically that, with the exception of glutamic acid, the rise in concentration of each of the amino-acids corresponded roughly to what would be expected when the appropriate amount of the protein hydrolysate was added to the fasting blood (see Figs 8 and 9).

Further suggestive evidence that the excess of amino-acids found in the portal blood represents the products of protein digestion and absorption is to be obtained by comparing the portal blood levels with those in systemic blood. Chromatographic analysis showed that in the casein experiment (Exp 2) the portal blood had definitely higher concentrations of amino acids than the systemic blood although there were no significant qualitative differences. The same result was obtained after feeding casein hydrolysate (Exp 7, and Figs 11 and 12) where there could be less doubt that absorption as amino-acids was taking place. These chromatographic results are supported by the more numerous and accurate quantitative analyses of Christensen (Addendum to this paper), who found that the total and free α -amino nitrogen was appreciably higher in portal than in systemic blood in all 1 and 2.5 hr specimens, although they were sometimes approximately equal in the 5 hr specimen when presumably the absorption from the gut was at an end.

The second group of amino acids, which occur in the blood, are interpreted as being compounds released by the tissues after the further metabolism of the common amino acids. This would account for the tendency to rise slowly in concentration during the 5 hr period. There is some evidence that

α aminobutyric acid is formed from methionine (Dent, 1947), presumably representing what is left behind after it has lost its methyl group and sulphur atom. γ -Aminobutyric acid is most likely to arise from the decarboxylation of glutamic acid although no direct evidence on this point has been obtained here or in the past except in experiments on putrefaction (Ackermann & Kutscher, 1910). It is difficult to draw conclusions from the behaviour of glutamine since this substance usually gives a distorted spot on the chromatograms. Moreover, the rough quantitative determinations of glutamine by the chromatographic method using the strength of the ninhydrin colour reaction do not give figures as high as have been reported for dog plasma by enzymic (Archibald, 1944) and by chemical (Hamilton, 1945) methods. The reasons for this discrepancy are not clear.

The amino acids of the third group are believed to play no part in the processes occurring during protein digestion.

The objection that the blood amino-acid levels are too low to account for the observed transference of nitrogen from one part of the body to another can be disposed of by a consideration of the volume of blood flow in question. In a normal dog weighing 10 kg the portal-plasma flow can be taken to be roughly about 200 ml/min (Burton-Oritz, 1911, Macleod & Pearce, 1914, Soskin, Essex, Herrick & Mann, 1938, Lipscomb & Crandall, 1947). Even at this rate of flow a concentration of 4 mg of amino nitrogen/100 ml can transfer several grams of nitrogen in the 5 hr span of an experiment. During digestion of a protein meal it is likely that a large increase in portal flow occurs which would be adequate to transfer all the nitrogen given in these experiments from the gut to the tissues in the form of amino acids.

No evidence was obtained that peptides can be absorbed in any appreciable amounts since none was found in the blood by the various chromatographic methods tried, even under the circumstances induced by the partial gastrectomy operation, which caused rises in total free amino nitrogen concentration of up to six times normal (see Addendum). The chemical results of Christensen (Addendum) do, however, indicate that about 1 mg/100 ml of 'bound α -amino nitrogen' is present in the blood and that this amount increases during the course of protein digestion. Similar rises after hydrolysis in the concentration of some amino-acids were also found by chromatography (Exp 12). There is considerable doubt as to the nature of this bound nitrogen. It may be due to peptides, but could equally well be from amino acids substituted, like the glycine in hippuric acid, on the α -amino nitrogen atom. There is indirect evidence for the existence of further examples of such acylated amino-acids. It

is interesting that in some of the experiments recorded here (e.g. Exps 7, 9 and 13) increases after hydrolysis in the amounts of free histidine and citrulline (and/or β -alanine) in the plasma were noted. In view of the definite identification of the latter as β alanine in the urine in one case (Exp 13) it may be that the substance increasing after hydrolysis is always β alanine, and if so it would be very likely that they both arise from the presence of carnosine in the blood. Carnosine can be detected by paper chromatography (Dent, 1948), but may be missed in low concentrations. Although it is a true peptide it could not arise from simple hydrolysis of a protein. A liberation of carnosine from the tissues into the blood, coincident with a rapid uptake from the blood by the tissue cells of free amino acids from the protein digestion, agrees with the views of Christensen, Streicher & Elbinger (1948). In guinea pigs amino acids appear to compete with each other for the means by which they are concentrated by the cell, with the result that the ability of tissue cells to hold one amino acid may decrease when other amino-acids are present in high concentrations in the blood. Such a rule might hold for a simple peptide like carnosine. Another reason for considering that the protein is not appreciably absorbed into the portal blood as peptides is based on the results of Exp 4. In this experiment the intestinal contents vomited by the dog 4-5 hr after giving the casein contained amino acids and very high concentrations of peptides, most of the amino nitrogen being in peptide form. The chemical analysis also done by Christensen on this sample confirmed this result. However, the portal-blood samples obtained at 2.5 and 5 hr and worked up and analyzed in exactly the same ways showed only the usual small amounts of bound amino nitrogen and no definite evidence of peptides. It would seem from this that the intestinal mucous membrane was acting as a very efficient barrier to the absorption of peptides. However, the finding of some 'peptides' in the urine when these were not detected in the blood (Exps 7, 9 and 13, Figs 13 and 15) does indicate the possibility of the absorption of small quantities. It was remarkable that the same four 'peptides' were found in the urine in the two experiments in which human-serum albumin was fed. The urinary concentration of blood peptides after the intravenous infusion of Amigen has been observed by Christensen *et al.* (1946).

With regard to the results found after ingestion of dog whole plasma protein it must first be emphasized that all the evidence obtained here has been negative in character. The homologous protein disappeared from the gut and no trace of it was found in the portal or systemic blood by the same methods as were applied in the experiments with heterologous proteins. Several explanations of this

can be advanced. Technical errors in the chromatographic analysis are not considered likely in view of the very definite results and of the many control samples taken. Moreover, the entirely independent chemical analyses of Christensen (Addendum) have given the same results in the case of Exp 6. We are left then with the following possibilities. First, that the protein is broken down to amino acids in the gut, and that the intestinal mucous membrane resynthesizes them into plasma protein, in which form they are then taken up by the portal blood. Secondly, the protein may be absorbed into the portal blood as large peptides, which, if of high molecular weight, would not be detected by the methods used. Thirdly, the protein is absorbed largely in an intact or undigested form.

The possibility of absorption of homologous intact plasma protein should be considered seriously, particularly in the light of present views upon the mechanism of particulate fat absorption and upon the passage of plasma proteins through cellular membranes (Madden & Whipple, 1940). It is interesting too to remark on the peculiar gut reaction which always occurred when the plasma was given. The tendency to diarrhoea and vomiting, not related to the strong salt concentration of the plasma (Exp 6), indicates that an abnormal state of affairs was induced in the intestine. Intact absorption of toxins and antitoxins from the gut in the adult animal can only occur after the mucous membrane has been damaged by simultaneous feeding of bile or of purgatives such as castor oil or aloes (Grasset, 1929). It was unfortunate that Exp 10, in which denatured homologous plasma was fed, failed because of the exceptionally severe vomiting, for it would have been difficult to imagine that such denatured protein could be absorbed intact. A serious difficulty in accepting any theory of intact absorption concerns the expected action of the gut enzymes. A preliminary experiment (unpublished work) in which dog plasma was incubated in turn with gastric and mixed intestinal juices obtained from other dogs, and then analyzed on paper chromatograms has shown conclusively that complete breakdown to free amino acids can result. If the experiments recorded here are to be reconciled with the theory of intact absorption it has to be assumed that this absorption takes place far more rapidly than the enzymic process or that the stimulus for secretion of the enzymes is lacking when homologous plasma is fed. A further objection exists, however, that cannot be readily disposed of. Clearly, if fed homologous plasma is absorbed intact then the nutritional result should be in every way identical with that obtained by giving the same amount intravenously, a fact which would be of possible clinical value. This, however, is not the case. Plasma protein given by vein to dogs

in nitrogen equilibrium produces a definite nitrogen retention lasting some days, while the same dose by mouth, however, produces the full amount of extra nitrogen in the urine (Holman, Mahoney & Whipple, 1934). No increased urinary output of sugar occurs in phlorrhizinized dogs after giving plasma by vein, although there is an increase after giving it by mouth (Howland & Hawkins, 1938). Yule & Hawkins (1941) later showed that a marked azotaemia occurred after feeding homologous plasma, good evidence that it was being metabolized. Similar differences in response to fed and injected plasma protein have been noted in human subjects (Forbes, Albright, Reifenshein, Bryant, Cox & Dempsey, 1948, Eckhardt, Lewis, Murphy, Batchelor & Davidson, 1948).

A possible explanation is that the plasma is associated with a nitrogen-retaining hormone, destroyed on passage through the liver and therefore only capable of exerting its action on the nitrogen balance when in the systemic, rather than in the portal, circulation. It is easier, however, to assume that the homologous protein is partly at least broken down in the gut before absorption, and that in this condition it cannot be resynthesized to the original protein. This would conform with modern views that the processes of synthesis and degradation of proteins are quite distinct and irreversible.

The fact that each of the dogs given homologous plasma had had a partial gastrectomy may be the explanation for the possible differences between these results and those of other workers.

The passage of intact homologous plasma protein through intestinal mucous membranes has long ago been demonstrated (Voit & Bauer, 1869, Heidenham, 1894). It is also well known that homologous plasma protein disappears when placed in many serous cavities in the body, e.g. peritoneal or pericardial (Hamburger, 1895).

At the present time it is not possible to decide in favour of any of the above mechanisms of homologous protein absorption from the gut. Further work is in progress on this subject as most of the theories suggested are open to direct experimental trial.

SUMMARY

1 Dog portal blood removed by means of the London (1935) cannula has been examined by paper chromatography at intervals after feeding various proteins. Jugular blood was also examined in some dogs. A partial gastrectomy operation had been performed on most of the dogs as this was shown to result in more rapid disappearance of the protein from the gut.

2 Under these conditions the ingestion of heterologous proteins such as casein, casein hydrolysate (Amigen), ground beef and human serum albumin,

resulted in large increases in the amino acid concentration of the portal blood. The jugular blood showed quantitatively smaller but qualitatively similar changes.

3 After ingestion of casein the rises in the concentrations of individual amino-acids in the portal blood paralleled closely those which could be brought about by adding the appropriate amount of casein hydrolysate to the fasting blood in an *in vitro* experiment. Glutamic acid, however, behaved in an exceptional manner for it was present in the portal blood to a much smaller extent than in the artificial mixture.

4 Peptides were not definitely found in the portal blood in any instance. Some evidence was obtained for the occasional presence of bound amino nitrogen of unknown identity. In some cases this was likely to have been in the form of carnosine.

5 After ingestion of human-serum albumin the urine contained excessive quantities of many amino-acids, especially of glutamic acid. There were also

several 'peptides'. Similar but less prominent changes in the urine were found after ingestion of Amigen.

6 The blood amino acids appeared to belong to three types: those which rose and fell characteristically after the protein meal, those which varied less regularly, tending if anything to rise slightly throughout the 5 hr period, and those which did not change appreciably during the experiment.

7 Dog whole plasma protein when given to the dogs by mouth did not cause any significant rise in portal amino acid concentration, and neither peptides nor bound amino nitrogen were found.

8 It is suggested that the homologous plasma protein was absorbed either intact or as large fragments, and that the heterologous proteins may have been largely if not entirely absorbed as free amino-acids.

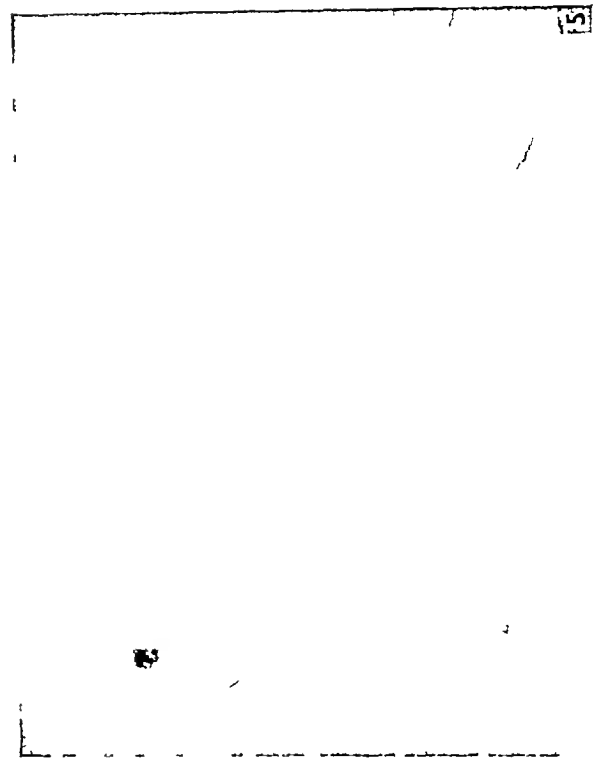
The authors gratefully acknowledge the help and encouragement given them by Dr G H Whipple and his department.

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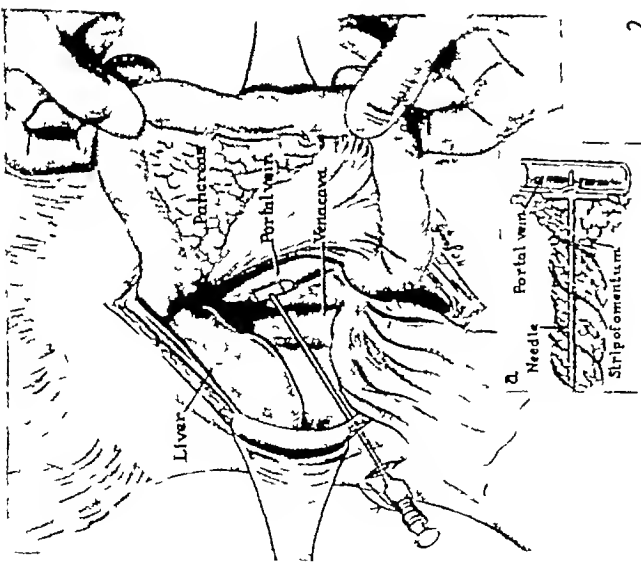
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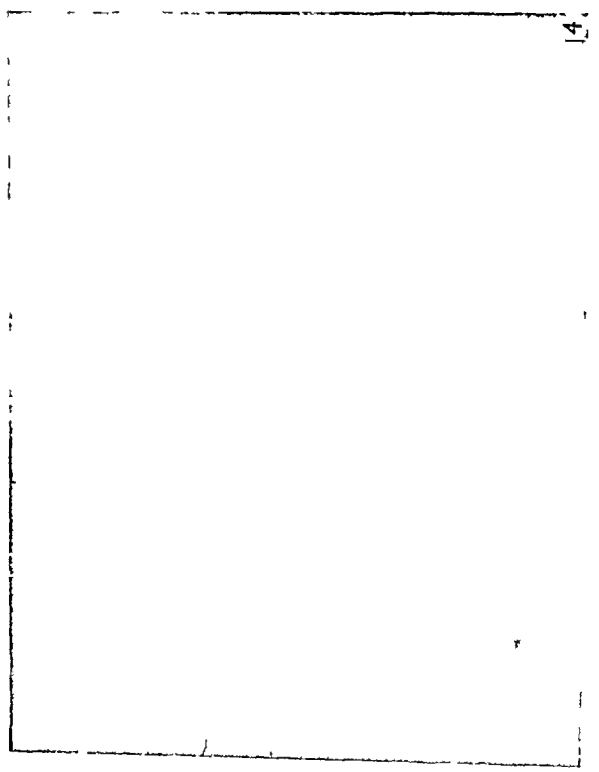
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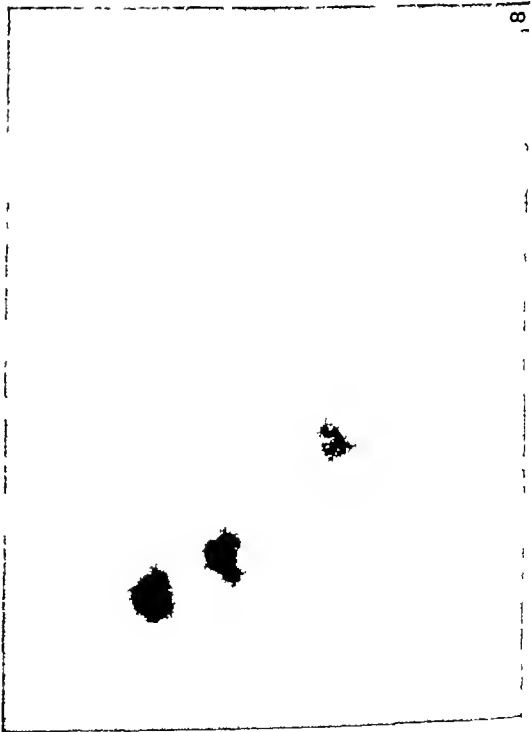
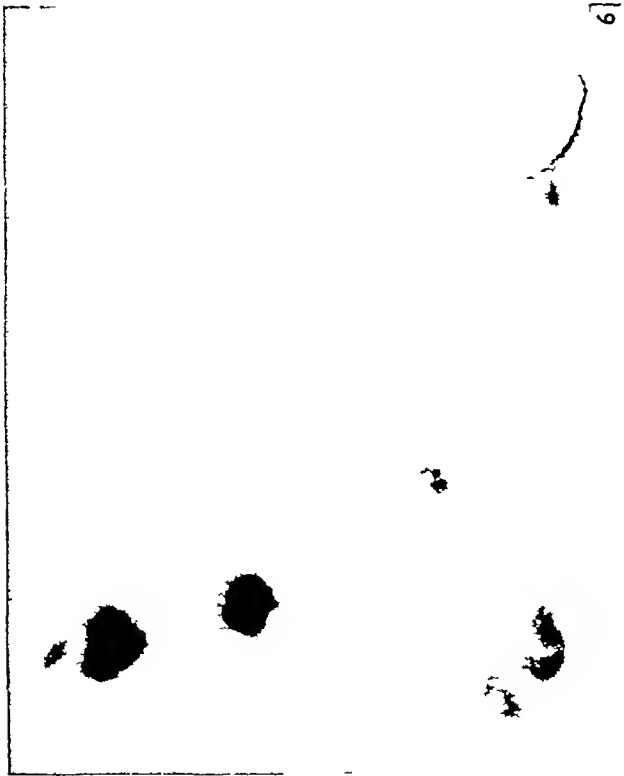
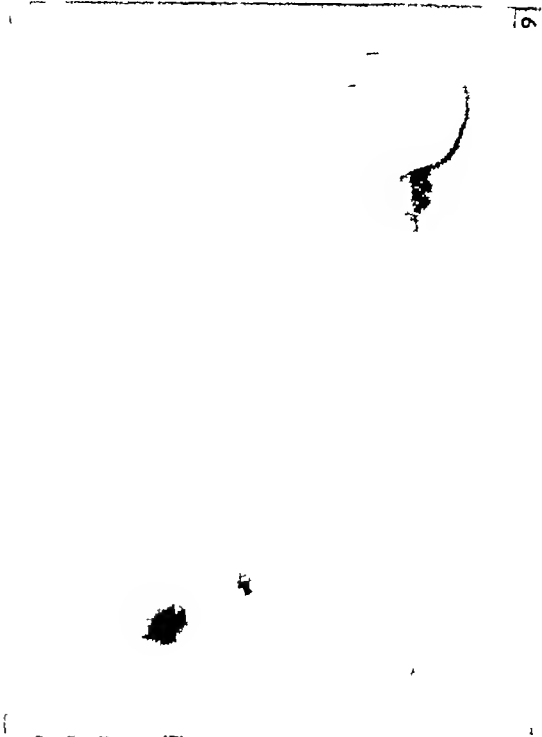
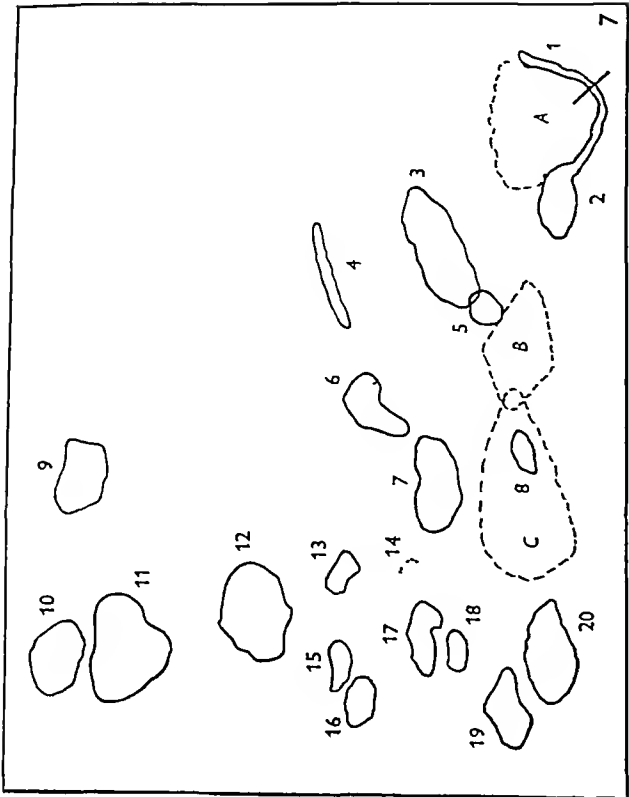


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4

C E DENT AND J A SCHILLING—STUDIES ON THE ABSORPTION OF PROTEINS
THE AMINO ACID PATTERN IN THE PORTAL BLOOD



C E DENT AND J A SCHILLING—STUDIES ON THE ABSORPTION OF PROTEINS
THE AMINO ACID PATTERN IN THE PORTAL BLOOD

EXPLANATION OF PLATES 4 AND 5

PLATE 4

Fig 2 Drawing showing the London cannula sutured in its final position to the portal vein. A strip of omentum is wrapped round the cannula, as shown in the inset, before the abdomen is finally closed. To withdraw portal blood the trocar is removed and a long needle inserted through the cannula until the wall of the vein is pierced.

Fig 3 X ray photograph of dog with cannula in position during an injection of thorotrast into the portal vein (Exp 5). The branches of the portal vein can be seen

weakly outlined. This confirmed that true portal blood samples had been taken in the experiment.

Fig 4 Photograph of chromatogram from a typical sample of fasting portal blood (Exp 13). (In this and subsequent photographs, standard exposures and routine of development have been imposed.)

Fig 5 Photograph of chromatogram from the portal blood 2.5 hr after feeding 500 g of ground beef (Exp 12).

PLATE 5

Fig 6 Photograph of chromatogram from the portal blood 2.5 hr after feeding 100 g of human albumin (Exp 13). This shows the highest concentration of amino acids seen in any of these experiments. It should be compared with fasting blood from the same experiment seen in Fig 4.

Fig 7 Key to Fig 6: 1=aspartic acid, 2=glutamic acid, 3=serine, 4=taurine, 5=glycine, 6=threonine, 7=alanine, 8=glutamine, 9=tyrosine, 10=phenylalanine, 11=leucine, 12=valine, 13= α -amino *n*-butyric acid, 14=histidine (this does not appear in Fig 6 but can be seen in Figs 5, 8 and 9), 15='fast-aminobutyric acid', 16=proline, 17=methionine sulfoxide, 18= γ -aminobutyric acid, 19=arginine, 20=lysine. In addition three unidentified substances, A, B and C, are shown, which appear, when outlined, as yellow areas. Substance A causes the serious distortion of aspartic and glutamic acids which are pushed apart in the upper region and

caused to run into one another below. The oblique line shows the approximate dividing line between the two amino acids as determined by the use of markers B and C usually overlap slightly, as shown. B causes the least trouble in practice, C, however, may overlie glutamine or push it slightly up or down. Serine and taurine and, to a less extent, threonine are also pulled into long streaks, presumably while they are moving with the yellow substance during the phenol run.

Fig 8 Photograph of chromatogram from the portal blood 2.5 hr after feeding 100 g of casein (Exp 5). This should be compared closely with Fig 9. See text for further discussion.

Fig 9 Photograph of chromatogram from fasting portal blood (Exp 5) with enough acid-hydrolyzed casein added to it to match the total amino nitrogen level of the sample shown in Fig 8.

Addendum. Conjugated Amino-acids in Portal Plasma of Dogs after Protein Feeding

By H. N. CHRISTENSEN, *The Children's Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts, U.S.A.*

(Received 27 April 1948)

The best available evidence for the absorption of important quantities of peptides during protein digestion came from the laboratory of Dr E. S. London from experiments upon dogs having cannulae affixed to various blood vessels (for references, see Christensen, Decker, Lynch, Mackenzie & Powers, 1947). The deficiencies in the analytical procedures used in this field have been discussed (Christensen *et al.* 1947). A re-examination of the question in dogs with cannulated portal veins has been made possible through the kindness of Dr C. E. Dent in supplying us with ultrafiltrates of

plasma obtained from dogs in experiments described in the preceding paper (Dent & Schilling, 1949). Analysis of these samples for α -amino nitrogen by the manometric ninhydrin procedure, before and after acid hydrolysis, showed measurable increases in the amino acid conjugates accompanied by very large rises in free amino acid concentrations.

EXPERIMENTAL

α -Amino N was determined in replicate by the manometric ninhydrin method at pH 2.5 (Hamilton & Van Slyke, 1943) upon samples of plasma ultrafiltrate before and after

hydrolysis by acid (Christensen & Lynch, 1946) In the case of dog 14, glutamine was also determined by the loss of α amino N upon prolonged heating (Hamilton, 1945) In this experiment the free and bound α amino N were determined at pH 4.7 (not pH 2.5) Glycine was determined by the formaldehyde released by ninhydrin (Alexander, Landwehr & Seligman, 1945)

DISCUSSION

Significant increases in the amino-acid conjugates of plasma were observed after the feeding of casein, ground beef and human serum albumin (Table 1)

digestion in these experiments were not necessarily fragments of the proteins fed, since the feeding of L glutamic acid to dogs (Table 1) or of glycine to humans (Christensen, Cooper, Johnson & Lynch, 1947) produced increases in the conjugates of plasma

A decrease in the glycine concentration of plasma, as well as a net decrease in the non-glycine, non glutamine amino acids, was observed during glutamate absorption (Table 1) Dent has observed decreases in other amino acids in the same samples by paper chromatography This chromatogram on 125 μ l of the 1 hr portal plasma ultrafiltrate

Table 1 *Amino-acids of portal and jugular plasma after feeding of protein and of glutamic acid*
(The values are in mg/100 ml of ultrafiltrate)

Dog no	Material fed	Type of α NH ₂ N	0 hr		1 hr		2.5 hr		5 hr	
			Portal	Jugular	Portal	Jugular	Portal	Jugular	Portal	Jugular
4	Casein (100 g)	Total	5.17	5.57	11.61	10.47	14.74	12.70	6.03	8.68
		Free	4.56	5.02	10.11	9.37	14.46	11.59	5.58	8.24
		Bound	0.61	0.55	1.5	1.1	0.3	1.1	0.45	0.44
5	Casein (100 g)	Total	4.84	4.07	16.5	13.0	18.9	12.2	10.4	11.56
		Free	4.41	3.88	13.4	10.80	16.3	9.30	10.2	9.06
		Bound	0.43	1.1	3.1	2.2	2.6	2.9	0.2	2.5
6	Canine plasma protein (100 g)	Free	4.10	3.53	3.04	3.06	2.60	3.18	3.24*	3.06*
12	Ground beef (500 g)	Total	6.6	5.4	6.73	5.6	16.3	10.9	14.90	8.88
		Free	5.84	4.64	5.84	4.62	14.8	—	13.75	8.32
		Bound	0.8	0.8	0.9	1.0	1.5	—	1.2	0.6
13	Human serum albumin (100 g)	Total	5.10	—	17.1	—	23.8	19.1	10.13	8.33
		Free	4.26	3.83	14.4	10.15	21.9	16.9	9.00	7.43
		Bound†	0.8	—	2.7	—	1.9	2.2	1.1	0.9
14	L Glutamic acid (as glutamate) (3 g/kg)	Glutamine	1.10	1.21	1.59	1.59	0.98	1.10	0.71	0.99
		Glycine	0.20	0.19	0.24	0.22	0.15	0.13	0.17	0.13
		Non glycine, non glutamine	2.69	2.79	0.99	0.88	4.46	3.80	2.24	2.02
		Bound	0.92	0.59	—	0.47	0.71	0.81	1.38	1.12

* These two samples were taken at 8 hr

† These values include no appreciable amount of acetyltrypophan The urine excreted during this test contained 342 mg/100 ml of free α amino N, which was 21 times the pre test concentration

These increases were far smaller, however, than those of free amino acids Furthermore, large and consistent portal jugular concentration differences were found in the free amino-acids but not in the conjugates, suggesting that the latter were perhaps entering the blood from the digestive tract much more slowly than were the free amino acids As a result of the partial gastrectomy, and the large size of the protein feeding, the ratio of the amount of protein to the amount of digestive enzymes present in the intestine was undoubtedly unusually large This might be expected to favour the absorption of intermediate degradation products of the proteins During digestion the intestine contained large quantities of peptides as indicated by the analysis of the vomitus of dog 4, obtained 4.5 hr after the test meal had been given Of the ultrafilterable amino acid nitrogen present, 84% was in peptide form, 16% free (Dent & Schilling, 1949)

The conjugates found in plasma during protein

showed, apart from the very large quantities of glutamic acid, only a slightly raised concentration of alanine, and just detectable traces of proline and of arginine All the other amino-acids were undetectable (Dent, private communication) These observations, together with similar observations in guinea pigs, will be discussed elsewhere (Christensen, Streicher & Elbinger, 1948)

SUMMARY

1 During the digestion of several protein meals by partially gastrectomized dogs, increases occurred in the concentrations of conjugated amino acids of portal and jugular plasma

2 The results indicate that at most a minor part of the protein was absorbed in peptide form The plasma conjugates were not necessarily fragments of the proteins fed since increases in plasma conjugates were also produced by feeding L glutamic acid

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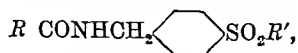
The Metabolism of Sulphonamides

6 THE FATE OF SOME *N*⁴-*n*-ACYL DERIVATIVES OF AMBAMIDE (MARFANIL) AND THE SULPHONE, V 335, IN THE RABBIT

BY R L HARTLES AND R T WILLIAMS, *Department of Biochemistry, University of Liverpool*

(Received 20 October 1948)

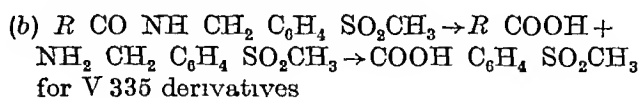
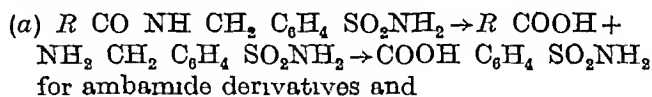
We have shown (Hartles & Williams, 1947) that the drugs ambamide (Marfanil) and V 335 lack systemic antibacterial activity because they are rapidly deaminized and oxidized to inactive metabolites. We suggested that the marked *in vitro* activity of these drugs might be preserved *in vivo* if the biologically labile $-\text{CH}_2\text{NH}_2$ group could be protected by a group which is removed in the body at a rate which allows therapeutic concentrations of the free drug to be attained. Acetylamamide (*p*-acetamidomethylbenzenesulphonamide), which has no antibacterial activity (Klarer, 1941), is however excreted unchanged (Hartles & Williams, 1947). The acetyl group certainly protects the $-\text{CH}_2\text{NH}_2$ group against deamination and oxidation, but is itself resistant to hydrolysis in the body. It does not follow, however, that, if the acetyl derivatives are resistant to hydrolysis *in vivo*, then other acylamino derivatives are also resistant. We have therefore synthesized and studied the fate of a number of *n*-acyl derivatives of the type



where $R \text{ CO}$ is a *n*-acyl group, and R' is $-\text{NH}_2$ in ambamide derivatives and $-\text{CH}_3$ in V 335 derivatives.

Not only have we searched for possible therapeutic agents among these compounds, but we have also noted a relationship between the chain length and the degree of hydrolysis of the acylamino group.

The extent of hydrolysis was assessed from the output in the urine of either *p*-carboxybenzenesulphonamide or *p*-methylsulphonylbenzoic acid and unchanged acyl derivatives, the reactions assumed to take place being



EXPERIMENTAL

Materials and methods

Synthesis of n acyl derivatives of ambamide and V 335
 With the exception of acetylamamide (Klarer, 1941, Bergem & Braker, 1944) and acetyl V 335 (Jensen, Schmith, Brandt, Lauritsen & Hanson, 1944) all the compounds now described are new and were prepared by the same general method.

p-Aminomethylbenzenesulphonamide, m.p. 153° (ambamide base), or *p*-aminomethylphenyl methyl sulphone, m.p. 85° (V 335 base), was heated with a molecular equivalent of the fatty acid for 30 min at 150–160°. The mixture was cooled, broken up and recrystallized from water (for formyl, propionyl and butyryl derivatives) or from ethanol (for C_6 – C_{18} acyl derivatives). The analytical data and melting points for these compounds are given in Table 1. All the compounds appeared white or colourless and the yields were 50–60% of theory.

Table 1 *Analytical data and melting points of N-n acyl derivatives of p aminomethylbenzenesulphonamide (ambamide) and p-aminomethylphenyl methyl sulphone (V 335)*

Compound	M p (°)	Found		Formula	Formula requires	
		C (%)	H (%)		C (%)	H (%)
Derivatives of ambamide						
Formylambamide* (white needles)	149	45.4	4.6	C ₈ H ₁₀ O ₃ N ₂ S	44.9	4.7
Acetylambamide†	177	—	—	—	—	—
Propionylambamide (plates)	144	49.65	6.0	C ₁₀ H ₁₄ O ₃ N ₂ S	49.6	5.8
Butyrylambamide (plates)	144	52.1	6.6	C ₁₁ H ₁₆ O ₃ N ₂ S	51.55	6.3
<i>n</i> Pentane 1 carbonylambamide (prisms)	173	54.4	7.1	C ₁₃ H ₂₀ O ₃ N ₂ S	54.9	7.1
<i>n</i> -Heptane 1 carbonylambamide (plates)	150	57.8	7.7	C ₁₅ H ₂₄ O ₃ N ₂ S	57.7	7.7
<i>n</i> Nonane 1 carbonylambamide (plates)	150	59.7	7.9	C ₁₇ H ₂₈ O ₃ N ₂ S	59.95	8.3
<i>n</i> Undecane 1 carbonylambamide (plates)	147.5	62.2	8.9	C ₁₉ H ₃₂ O ₃ N ₂ S	61.9	8.75
<i>n</i> Tridecane 1 carbonylambamide (plates)	145.5	63.0	9.1	C ₂₁ H ₃₆ O ₃ N ₂ S	63.6	9.15
<i>n</i> Pentadecane 1 carbonylambamide (prisms)	147.5	64.6	9.5	C ₂₃ H ₄₀ O ₃ N ₂ S	65.05	9.5
<i>n</i> Heptadecane 1 carbonylambamide (plates)	151.5	66.3	9.95	C ₂₅ H ₄₄ O ₃ N ₂ S	66.3	9.8
Derivatives of V 335						
Formyl V 335* (plates)	134	50.4	5.25	C ₈ H ₁₁ O ₃ NS	50.7	5.2
Acetyl V 335†	125	—	—	—	—	—
Propionyl V 335 (plates)	150	54.4	6.2	C ₁₁ H ₁₅ O ₃ NS	54.75	6.3
Butyryl V 335 (plates)	122	56.7	6.8	C ₁₂ H ₁₇ O ₃ NS	56.45	6.7
<i>n</i> Pentane 1 carbonyl V 335 (plates)	125	59.3	7.6	C ₁₄ H ₂₁ O ₃ NS	59.3	7.5
<i>n</i> Heptane 1 carbonyl V 335 (plates)	130	61.75	8.3	C ₁₆ H ₂₅ O ₃ NS	61.7	8.1
<i>n</i> Nonane 1 carbonyl V 335 (plates)	133	63.0	8.3	C ₁₈ H ₂₉ O ₃ NS	63.7	8.6
<i>n</i> Undecane 1 carbonyl V 335 (plates)	133.5	65.7	9.4	C ₂₀ H ₃₃ O ₃ NS	65.35	9.05
<i>n</i> Tridecane 1 carbonyl V 335 (plates)	130.5	66.8	9.4	C ₂₂ H ₃₇ O ₃ NS	66.8	9.45
<i>n</i> Pentadecane 1 carbonyl V 335 (plates)	133.5	68.1	9.8	C ₂₄ H ₄₁ O ₃ NS	68.05	9.75
<i>n</i> -Heptadecane 1 carbonyl V 335 (plates)	108	69.25	10.0	C ₂₆ H ₄₅ O ₃ NS	69.1	10.05

* These were presented to us by R. F. Reed, Ltd., Barking, and are described here for the first time with their permission.

† These acetyl derivatives are included for completeness.

Animals and administration of drugs Chinchilla rabbits (2.5–3.0 kg.) maintained on a diet of 50 g. Lever cubes and 200 g. cabbage daily were used throughout. The compounds (1 g. doses) were suspended in water and administered by stomach tube. Urines were collected daily and analyzed for unchanged material and for *p*-carboxybenzenesulphonamide or *p*-methylsulphonylbenzoic acid. With the *n*-pentane-1-carbonyl and higher derivatives the faeces were also examined for unchanged compounds.

Estimation of the carboxylic acids in urine The sample of filtered urine (c. 100 ml.) was acidified with 1/10 vol. of conc. HCl and extracted continuously with ether for 3 hr. The extract was evaporated and the residue dissolved in 2N NaOH. After filtration, this solution was acidified with conc. HCl, kept at 0° for 1 hr. and the precipitated carboxylic acids filtered into a tared Gooch crucible, dried and weighed.

Isolation of unchanged compounds from urine (a) *Procedure for formyl, acetyl and propionyl derivatives* The filtered urine sample (c. 100 ml.) was saturated with (NH₄)₂SO₄ and then extracted four times with half a volume of methyl ethyl ketone. The solvent was distilled from the extract and the residue recrystallized from hot water, filtered, dried and weighed.

(b) *Procedure for butyryl and higher derivatives* The filtered urine sample was extracted with ether continuously for 3 hr. A longer period did not increase yields. The extract was evaporated and the residue was recrystallized

from water or aqueous ethanol and the crystals were filtered, dried and weighed.

Isolation of unchanged compounds from faeces Faeces, collected for 5 days after feeding the drugs, were ground with anhydrous Na₂SO₄ and extracted with acetone in a Soxhlet apparatus. The acetone was removed and the dry residue was dissolved in the minimum of hot absolute ethanol. The acyl derivative present was precipitated with light petroleum (b.p. 40–60°) and filtered off. After recrystallization from 90% ethanol, the compound was dried and weighed.

RESULTS

Table 2 gives the detailed results for two compounds. It should be noted that the urinary excretion of metabolites of the formyl, acetyl, propionyl and butyryl compounds was complete in 24 hr. after dosing, whereas for the *n*-pentane-1-carbonyl and higher derivatives a 48 hr. collection was necessary. Tables 3 and 4 give the average results for all the derivatives studied. Table 5 gives the approximate percentage of the dose of the *n*-pentane-1-carbonyl and higher derivatives of ambamide and V 335 eliminated in the faeces collected for 5 days after dosing. The lower derivatives were completely absorbed and none appeared in the faeces.

Table 2 *The urinary excretion of metabolites by rabbits receiving the formyl and n pentane-1-carbonyl derivatives of ambamide*

Compound fed	Rabbit no	Wt (kg)	Dose (g)	Urine vol (ml)	Sample analyzed (ml)	Un-changed compound recovered in sample (mg)	p Carboxy benzene sulphonamide in sample (mg)	Percentage of dose excreted	
								Un-changed	Oxidized
Formylambamide	108	2.5	1	310	100	240	9.4	71.1	3.0
	109	2.3	1	360	100	210	4.5	73.2	1.7
	112	2.6	1	270	100	238	5.4	63.0	1.5
n Pentane 1 carbonylambamide	91	3.0	1	(i)* 170	160	0	143	0	49.6
				(ii) 160	145	0	191		
	99	2.7	1	(i) 165	150	0	232	0	39.5
				(ii) 115	105	0	25		
	114	3.2	1	(i) 155	135	0	194	0	44.6
				(ii) 140	130	0	95		

* The metabolites of n pentane 1 carbonylambamide are excreted during 2 days

Table 3 *The urinary excretion of metabolites of n acyl derivatives of ambamide in the rabbit*(Formula of compounds $R \text{ CONHCH}_2\text{C}_6\text{H}_4\text{SO}_2\text{NH}_2$ Dose 1 g /animal)

Ambamide derivative fed	R	No of experiments	Percentage of dose excreted	
			Unchanged	As p carboxy-benzene sulphonamide
(Ambamide)	—	3	(0)*	(84)*
Formyl	H	3	69.1	2.1
Acetyl	CH ₃	3	54.6	0
Propionyl	C ₂ H ₅	3	62.1	0
Butyryl	C ₃ H ₇	3	41.9	20.8
n Pentane 1 carbonyl	C ₅ H ₁₁	3	0	44.6
n Heptane 1 carbonyl	C ₇ H ₁₅	2	0	43.5
n Nonane 1 carbonyl	C ₉ H ₁₉	3	0	15.3
n Undecane 1 carbonyl	C ₁₁ H ₂₃	2	0	0
n Tridecane 1 carbonyl	C ₁₃ H ₂₇	2	0	0

* These figures are quoted from Hartles & Williams (1947)

Table 4 *The urinary excretion of metabolites of n-acyl derivatives of V 335 in the rabbit*(Formula of compounds $R \text{ CONHCH}_2\text{C}_6\text{H}_4\text{SO}_2\text{CH}_3$ Dose 1 g /animal)

V 335 derivative fed	R	No of experiments	Percentage of dose excreted	
			Unchanged	As p methylsulphonylbenzoic acid
(V 335)	—	—	(0)*	(87)*
Formyl	H	3	48.5	15.8
Acetyl	CH ₃	3	61.1	0
Propionyl	C ₂ H ₅	3	42.9	13.1
Butyryl	C ₃ H ₇	3	3.0	62.1
n Pentane 1 carbonyl	C ₅ H ₁₁	3	0	56.8
n Heptane 1 carbonyl	C ₇ H ₁₅	2	0	41.0
n Nonane 1 carbonyl	C ₉ H ₁₉	3	0	22.2
n Undecane 1 carbonyl	C ₁₁ H ₂₃	2	0	0
n Tridecane 1 carbonyl	C ₁₃ H ₂₇	2	0	0

* These figures are quoted from Hartles & Williams (1947)

Table 5 *Faecal excretion of n-acyl derivatives of ambamide and V 335 by rabbits*

Compound fed	Percentage of dose excreted unchanged in the faeces in 5 days
Butyrylambamide	0
<i>n</i> Pentane 1 carbonylambamide	5
<i>n</i> Heptane 1 carbonylambamide	10-15
<i>n</i> -Nonane 1 carbonylambamide	30
<i>n</i> Undecane 1 carbonylambamido	40
<i>n</i> Tridecane-1 carbonylambamide	50
Butyryl V 335	0
<i>n</i> Pentane 1 carbonyl V 335	2-3
<i>n</i> Heptane 1-carbonyl V 335	10
<i>n</i> -Nonane 1-carbonyl V 335	40
<i>n</i> Undecane 1-carbonyl V 335	50
<i>n</i> Tridecane 1 carbonyl V 335	50

Injection of butyryl V 335 The derivatives higher than butyryl were not soluble enough to be injected

Butyryl V 335 (500 mg) was dissolved in 15 ml of physiological saline solution at 40° and injected subcutaneously into a 3 kg rabbit. The urine was examined each day for the following 3 days for the unchanged drug and *p*-methylsulphonylbenzoic acid. No unchanged drug was excreted, but the acid was found on the first and second days (yields 100 and 50 mg respectively), but not on the third day.

Thus butyryl V 335 on injection is converted mainly to *p*-methylsulphonylbenzoic acid as is found on oral administration (see Table 4), although excretion of the acid takes longer after injection than after feeding.

DISCUSSION

The present work shows that the extent of deacylation and subsequent deamination and oxidation of the *p*-acylaminomethylsulphones depends on the chain length of the acyl group. This conclusion is illustrated in Fig. 1, in which the number of carbon atoms in the acyl group is plotted against the ratio (oxidized compound) $\times 100 / (\text{oxidized} + \text{unchanged compound})$.

Free ambamide and V 335 are excreted entirely oxidized. Formylambamide is only slightly deacylated and oxidized, the main bulk being excreted unchanged in the urine. Formyl and propionyl V 335, however, do undergo deacylation, for about 25% of the absorbed compounds is excreted as *p*-methylsulphonylbenzoic acid and 75% is unchanged. Both acetyl derivatives and propionylambamide are excreted entirely unchanged. With the higher derivatives (butyryl, *n*-pentane-, *n*-heptane- and *n*-nonane-1-carbonyl) of V 335, the absorbed part is excreted entirely deacylated and oxidized. Butyrylambamide is deacylated and oxidized to the extent of 33%, the rest of the absorbed drug being excreted unchanged. The higher

derivatives of ambamide are not completely absorbed, but that which is absorbed is entirely deacylated and oxidized. As the chain lengths of derivatives increase beyond butyryl, absorption of the drugs becomes progressively less (Fig. 2) and much of the unchanged drug is eliminated in the faeces. The compounds with a chain length of more than 10 carbon atoms are not absorbed at all.

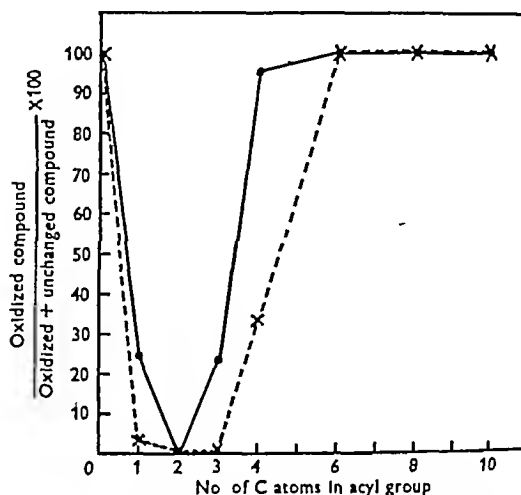


Fig. 1 The relation between the extent of deacylation in the rabbit of *N*-*n* acyl derivatives of ambamide and of V 335 and the number of carbon atoms in the acyl group *R* CO \times - - \times , ambamide derivatives, \bullet — \bullet , V 335 derivatives

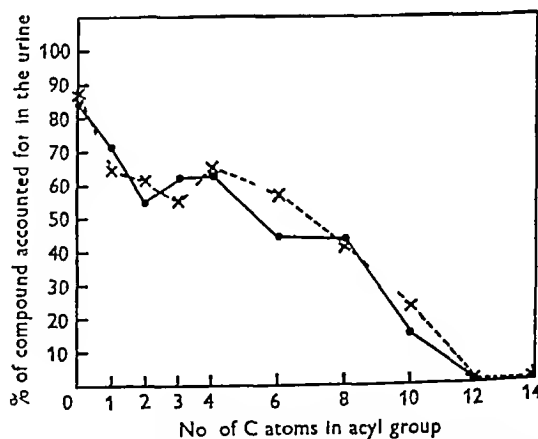


Fig. 2 Graph showing the percentage of *N*-*n* acyl derivatives of ambamide and V 335 absorbed and oxidized by rabbits, plotted against the number of carbon atoms in the acyl group *R* CO \times - - - \times , ambamide derivatives, \bullet — \bullet , V 335 derivatives

It is clear from these experiments that, beyond the acetyl and propionyl derivatives, the longer the chain the easier is the deacylation and consequent oxidation. These results are similar to those of Kohl & Flynn (1940) who found that *in vitro* deacylation by rat liver extract of *N*⁴ *n* acylsulphanil amides occurred more readily as the chain length

increased This is not true, however, for acylsulphamezathines (Krebs, Sykes & Bartley, 1947)

It is also interesting to note that neither of the acetyl derivatives are deacetylated by the rabbit This inability of the rabbit to split the acetamido group to any great extent has now been observed in this laboratory with a number of compounds of the type $ArNHC(=O)CH_3$, where Ar represents an aryl group (e.g. acetanilide, *o*- and *p*-acetamidophenols, phenacetin, *p*-acetamidobenzoic acid, *p*-acetamidophenylacetic acid, acetylsulphanilic acid and N^4 acetylsulphanilamides) or $ArCH_2NHC(=O)CH_3$ (e.g. *p*-hydroxybenzylacetamide, acetylamamide and acetyl V 335) (Smith & Williams, 1948, Hartles & Williams, 1948)

Krebs *et al* (1947) point out that enzymes splitting N^4 acylsulphonamides belong to the group called 'acylases' by Abderhalden & von Ehrenwall (1931) These enzymes split the peptide link in compounds of the type $RCONHR'$ In the compounds studied here R is a *n*-alkyl group and R' is either $-CH_2C_6H_4SO_2NH_2$ (ambamide derivatives) or $-CH_2C_6H_4SO_2CH_3$ (V 335 derivatives) Splitting of the acylamino group in the intestine before absorption is ruled out because only unchanged compounds were found in the faeces

It was not expected that any of these acyl derivatives would have anticlostridial activity *in vitro*, because of blocking of the amino group This expectation was confirmed by the antibacterial test quoted in the Appendix It is clear from Fig 1 that formyl, propionyl and butyryl V 335 and butyrylamamide are compounds which are possible therapeutic agents *in vivo*, for all four are deacylated *in vivo* and therefore there is the possibility that free V 335 and ambamide may be released in the tissues in therapeutic concentrations if deamination is not too rapid These four compounds were tested *in vivo* for antibacterial activity, against a *Clostridium septicum* infection in mice (see Appendix) Although they were administered orally in doses of 1 g/kg every 4 hr

they had no permanent curative effect, but they did show some activity in the earlier hours of the treatment Dr Henderson suggested that although the drugs might show greater effectiveness in larger doses and with extended treatment, continuation of the trials was not justified in view of the much greater effectiveness, for example, of sulphapyridine

SUMMARY

1 The extent of deacylation and subsequent deamination and oxidation of a series of *N*-*n*-acyl derivatives of *p*-aminomethylbenzenesulphonamide (Marfanil or ambamide) and of *p*-aminomethylphenyl methyl sulphone (V 335) has been studied in the rabbit

2 Formyl-, acetyl- and propionyl-ambamide are largely excreted unchanged in the urine Acetyl V 335 is similarly excreted unchanged

3 Butyrylamamide and formyl, propionyl and butyryl V 335 are partly deacylated and oxidized and partly excreted unchanged in the urine

4 As the chain length of the acyl group increases from C_6 , the amount of the acyl derivatives of ambamide and V 335 absorbed becomes progressively less and at C_{12} none is absorbed and the compounds appear in the faeces That part of the C_6 and higher derivatives which is absorbed is, however, completely deacylated and oxidized

5 Twenty new derivatives of ambamide and V 335 have been synthesized, and a selection of them showed no activity *in vitro* against *Cl welchii*

6 The *in vivo* therapeutic possibilities of the compounds studied have been discussed

The authors wish to thank R. F. Reed Ltd., Barking, for generous supplies of ambamide, V 335 and their formyl derivatives, and Drs A. Spinks (Imperial Chemical Industries Ltd.) and D. W. Henderson (Microbiological Research Department, Porton) for the antibacterial tests The expenses of this work were in part defrayed by a grant from the Medical Research Council

Appendix Antibacterial Testing of Ambamide and V 335 Derivatives

In vitro tests A selection of the compounds described above was tested for antibacterial activity against *Mycobacterium tuberculosis* and *Clostridium welchii* The testing was arranged by Dr A. Spinks who sent us the report shown in Table 6

In the tests on *Myco tuberculosis* the index $<1/1$ represents no effect at all at 1/1000 dilution The index $<1/3$ means incomplete inhibition at 1/1000 dilution, but some partial inhibition down to 1/9000 The indices 1/4 and 1/5 mean partial inhibition down to dilutions of 1/27,000 and 1/81,000

respectively It is clear that formyl derivatives show more inhibition than the others

In vivo tests The four compounds which, as Fig 1 suggests, may have therapeutic activity *in vivo* were tested against an infection with *Cl septicum* in mice by Dr D. W. Henderson* of the Microbiological Research Department, Porton Dr Henderson reports as follows 'The route of infection was the mildest we know of, and the number of lethal doses kept to a low level None of

* These results are published with the permission of the Chief Scientist, Ministry of Supply

these compounds would seem to compare with, for example, M and B 693. There is just a suggestion that they had some effect because at the 12th hour (see Table 1) the controls showed definite signs of illness, whereas the mice under treatment appeared quite well. By the 24th hour there is no therapeutic effect evident.

Table 6

Compound	Activity	
	<i>Myco tuberculosis</i>	<i>Cl welchii</i>
Formylambamide	<1/4	Inactive
Butyrylambamide	<1/1	"
n-Heptane 1-carbonylambamide	<1/1	"
n-Tridecane 1-carbonylambamide	<1/1	"
n-Heptadecane-1-carbonylambamide	<1/1	"
Formyl V 335	<1/5	"
Acetyl V 335	<1/1	"
Propionyl V 335	<1/3	"
n Undecane 1 carbonyl V 335	<1/1	"
n Pentadecane 1 carbonyl V 335	<1/1	"

Table 7 *The effect of formyl, propionyl, and butyryl V 335 and butyrylambamide on an infection with Clostridium septicum in mice*

(Mice Weight, 18–20 g, 10 mice in each group. Infection about 10 minimal infecting doses (50 spores), 0.05 ml saline spore suspension + 0.05 ml. 5% aqueous CaCl₂ mixed immediately before injection intradermally into shaved abdomen. Drugs 20 mg of the drug suspended in 0.5 ml saline given by mouth at each dose, (i) 1 hr before infection, (ii) 4 hr after infection, (iii) 8 hr after infection and (iv) 12 hr after infection.)

Drug given	State of animals					
	12 hr after infection		24 hr after infection		48 hr after infection	
	Sick	Dead	Sick	Dead	Sick	Dead
Controls (no drug)	7/10	0/10	2/10	8/10	—	10/10
Formyl V 335	0/10	0/10	3/10	7/10	—	10/10
Propionyl V 335	0/10	0/10	1/10	9/10	—	10/10
Butyryl V 335	0/10	0/10	—	10/10	—	—
Butyrylambamide	0/10	0/10	1/10	9/10	—	10/10

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The Fluorimetric Estimation of Riboflavin in Foodstuffs and other Biological Material

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Various methods have been published for the estimation of riboflavin (Hodson & Norris, 1939, Conner & Straub, 1941, Andrews, 1943, Barton-Wright & Booth, 1943, Slater & Morell, 1946, Morell & Slater, 1946, Morell, 1947). Some of them employ adsorption of the vitamin on activated surfaces and its subsequent elution. The disadvantages of the adsorption techniques are that they are tedious, and may result in serious losses because of incomplete adsorption or elution of riboflavin (Rubin & DeRitter, 1945). Since 1942 we have used with satisfactory results the method described below, which, by using a sensitive photocell-galvanometer circuit, enabled us to estimate the vitamin

directly without concentrating it by adsorption. A similar technique has been described by Rubin, DeRitter, Schuman & Bauernfeind (1945) and by Scott, Hill, Norris & Heuser (1946).

EXPERIMENTAL

Principle of method

The main features of our technique are (1) Extraction on the water bath with 0.1 N-HCl. Precipitation of proteins by metaphosphoric acid, and/or digestion with takadiastase when starchy materials are assayed.

(2) Washing of the extract with chloroform at acid pH, and oxidation with saturated potassium permanganate solution followed by treatment with hydrogen peroxide to decolorize the remaining permanganate

(3) The extracts are adjusted to pH 6, and methanol added to clarify the extract if necessary

(4) Fluorimetric readings are taken using a blue filter for the incident light and a yellow filter between the solution and photocell. A sensitive photocell galvanometer circuit is used

(5) In the blank, the riboflavin is reduced to a non-fluorescent compound with a neutralized solution of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$)

Reagents

HCl (0.1N), NaOH (20%, w/v), acetic acid (1%, w/v), metaphosphoric acid (25%, w/v) freshly prepared, KMnO_4 (4%, w/v), H_2O_2 w/v (3%, w/v), chloroform, methanol, light petroleum, acetone 0.2N H_2SO_4 solution (1 l), sodium dithionite solution 0.5 g $\text{Na}_2\text{S}_2\text{O}_4$, 0.6 g NaHCO_3 dissolved in 10 ml water, prepared immediately before use Takadiastase (Parke, Davis and Co)

Riboflavin standard Riboflavin (25 mg) in 50 ml distilled water (at 50°) + 1 ml glacial acetic acid. Mix with a further 500 ml distilled water (at 50°), stir until completely dissolved, and make up to 1 l with distilled water and ethanol to a final concentration of 20%. The solution was kept in a dark brown bottle in the refrigerator. A dilute standard solution, containing 5 μg /ml, was prepared each week by diluting 10 ml of the strong riboflavin standard to 50 ml with 20% ethanol acidified with 1 drop of conc HCl

Apparatus

Single photocell fluorimeter (Cohen type), connected with a sensitive galvanometer (Cambridge Instrument Co., short period galvanometer, 460 Ω resistance at 20°, external resistance for critical damping 2800 Ω , 4.4 sec period, 4600 mm deflexion for 1 μamp at 1 m. The deflexion was doubled by placing the scale at a distance of 2 m). A photocell was chosen with a high sensitivity and a suitable inner resistance to obtain the critical damping of the galvanometer. The light source was a Hewittic Ulvianc Therapeutic Lamp, a.c. burner, 3500 c.p., with a suitable optical arrangement

The Hilger fluorimeter can be used as a Cohen type instrument by connecting the galvanometer directly with the right-hand cell as described by Bolton (1944)

The following filters were used Calorex and Wratten no 47A (blue) between the light source and solution, Flavazin filter Wratten no 16 between the solution and photocell

Preparation of extracts

Different extraction procedures have to be used depending on the material examined. The extraction procedures should be carried out in a semi-darkened room

(a) For foodstuffs rich in proteins

When dried eggs are assayed, a preliminary treatment is necessary. Wash the weighed sample of the egg powder with

light petroleum, centrifuge, repeat washing, centrifuge, evaporate off the remaining light petroleum on a water bath and proceed as below with the HCl extraction

Heat a weighed ground sample of the foodstuff (containing about 15 μg of riboflavin) with 50 ml of 0.1N-HCl on a boiling water bath for 30 min. Cool and add 5 ml of 25% metaphosphoric acid. Mix, wait 10 min, then centrifuge. Decant the supernatant fluid. Re-extract the residue with 35 ml of 1% acetic acid for 10 min on the water bath. Centrifuge and unite the liquid with the previous extract. Wash residue with 10 ml of 1% acetic acid (no heating necessary) and centrifuge. Combine the extracts and make up to 100 ml with water. Wash a sample (30–50 ml) with an equal volume of chloroform in a separating funnel. Centrifuge to hasten the separation of the layers. Oxidize 25 ml of the clear aqueous layer by adding a 4% solution of KMnO_4 drop by drop with continuous stirring. Allow the pink colour to disappear completely before adding the next drop. Continue the additions until a faint pink lasts for 30 sec. Decolorize the excess KMnO_4 with 1–2 drops of a 3% H_2O_2 solution. Wait 5 min, then neutralize to pH 5–6.0 with 2% NaOH (i.e. yellow to bromothymol blue and blue to bromocresol green, used as external indicators). Make up to 75 ml with water. Centrifuge if necessary

(b) For cereals and foodstuffs containing starch

Use 5–10 g of material for extraction. Add 50 ml of 0.1N HCl and, stirring continuously, bring the mixture to the boil. Continue with the extraction as described above, omitting the metaphosphoric acid. Centrifuge while warm. Bring the combined supernatants to pH 4.5 with 20% NaOH (bromocresol green as external indicator). Digest the solution with 10–20 mg of takadiastase (Parke, Davis and Co) for 2 hr at 37° in an incubator in the presence of a few drops of sulphur-free toluene. Adjust the volume to 100 ml. Centrifuge the digested extract. Add to a sample of the supernatant fluid 4 drops of conc HCl and wash with an equal volume of chloroform in a separating funnel. Proceed as above by taking 30 ml of the washed extract for oxidation. After adjusting the pH of the solution to 5.5–6.0, add 10 ml of methanol and bring the volume to 50 ml with water. Centrifuge for a short time if a precipitate has formed

(c) Acetone H_2SO_4 (1:1) extraction, suitable for cereals and vegetables

An alternative extraction procedure has been devised which gives comparable results with the extraction procedure (b), and can be used in some samples where extraction (b) does not result in clear extracts

Reflux 5 g of the sample with 50 ml of the acetone 0.2N- H_2SO_4 solution on a water bath for 30 min. Cool and centrifuge. Decant off the liquid and return the residue to the flask with 35 ml of fresh acetone H_2SO_4 solution. Reflux for a further 15 min. Centrifuge, and wash the residue with 15 ml of fresh acetone H_2SO_4 solution. Combine the supernatant fluids and evaporate off the acetone completely *in vacuo* at about 60°. Dilute the residual aqueous solution to 50 ml, wash with chloroform, and proceed as under (b)

Notes The takadiastase preparation should be tested for the presence of appreciable amounts of riboflavin, and if necessary the proper correction should be made. It is also advisable to check the reagents, especially methanol, for a

possible blank fluorescence. The final extracts should be water clear even under inspection in ultraviolet light, otherwise quenching will interfere with the accuracy. Auto claving with 0.05N HCl is a possible alternative extraction procedure, but extraneous colours were observed with certain foodstuffs which decreased the reliability of this procedure.

Measurement of fluorescence

Add to glass cell *A* 0.2 ml water, and to glass cell *B* 0.2 ml (1 μ g) of the dilute riboflavin standard. Measure 17 ml of the unknown solution into each of the two glass cells *A* and *B*. Take at least three alternate readings in quick succession on both solutions by moving the sliding carrier into position. Then add 0.2 ml of a freshly prepared $\text{Na}_2\text{S}_2\text{O}_4$ solution to glass cell *A*, stir and take readings again. As the reduction may take 30–60 sec to complete take readings until repeats agree. This last reading gives the value for the blank.

Calculation

$$\text{Riboflavin} = \frac{Q_1 - B}{Q_2 - Q_1} \times \frac{V_3}{17} \times \frac{V_1}{V_2} \times \frac{1}{S} \mu\text{g/g},$$

where S = amount of sample taken (g or ml), Q_1 = galvanometer reading in mm of the unknown solution (i.e. glass cell *A* before reduction), Q_2 = galvanometer reading in mm of the unknown solution + 1 μ g of riboflavin (i.e. glass cell *B*), B = galvanometer reading in mm of the blank (i.e. glass cell *A* after reduction), V_1 = vol of combined aqueous extracts, V_2 = vol of aqueous extract taken for oxidation, V_3 = vol. of final solution prepared for fluorimetry.

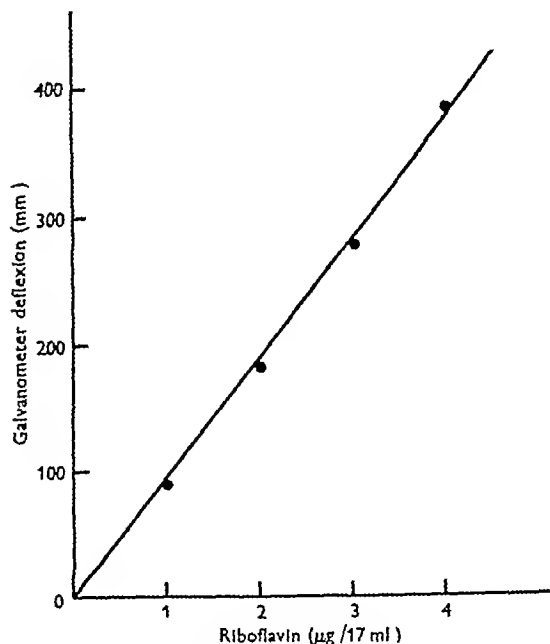


Fig 1 Standard curve for pure riboflavin

Sensitivity of method and standard reference curve

We have found that 1 μ g of riboflavin in 17 ml of 0.05N phosphate acetic buffer of pH 6 gives a maximum galvanometer deflexion of 182 mm, by closing the adjustable slit which decreases the exciting light, we normally work with a deflexion of 90 mm for 1 μ g/17 ml of solution.

Fig 1 shows the response curve. There is a straight-line relationship between galvanometer readings and concentration of riboflavin up to at least 5 μ g/17 ml of solution.

Reproducibility

To check the reproducibility of the method, several samples were analyzed in 8–12 replicates (Table 1). The coefficient of variation ranged from 2.2 to 4.6%. The

Table 1 Reproducibility of results

Material	No of estimations	Mean ($\mu\text{g/g}$)	Riboflavin	
			Standard error of single determination ($\mu\text{g/g}$)	Coefficient of variation (%)
Yeast, dried	8	50.2	± 1.082	2.2
Meat, dehydrated	8	6.92	± 0.321	4.6
Soya bean	12	3.72	± 0.171	4.6

Table 2 Riboflavin content of various foods

Material	No of samples (different sources)	Riboflavin ($\mu\text{g/g}$)	
		Mean	Range
Beef, fresh	3	1.6	1.5–1.7
Beef, dehydrated	1	6.2	—
Beef, corned	2	2.0	1.8–2.2
Pork, fresh	2	2.4	2.1–2.7
Pork, dehydrated	3	4.5	4.3–4.8
Liver, calf	6	13.5	11.8–14.6
Liver, sheep	10	29.4	21.6–36.0
Liver, guinea pig	1	40.0	—
Eggs, dried	10	13.8	8.2–15.3
Milk, fresh, cow's	3	1.5	1.4–1.8
Milk, human	6	0.6	0.4–0.7
Wheat flour, 100% extraction	5	1.2	1.05–1.35
Rye, flour mixed, whole	1	2.7	—
Oatmeal	1	1.0	—
Sussex oats, ground	1	1.5	—
Maize meal, yellow	1	0.8	—
Soya bean, Manitoba brown	1	3.7	—
Soya flour, low fat	1	7.2	—
Soya flour, high fat	1	12.0	—
Peanuts	1	2.8	—
Potatoes, fresh	4	0.33	0.29–0.39
Potatoes, peeled, dehydrated	1	1.3	—
Doon Star	1	1.5	—
King Edward	1	0.6	—
Majestic	1	0.5	—
Tomatoes	3	0.55	0.42–0.76
Carrots	1	0.27	—
Carrot puree	1	1.4	—
Pea puree	2	0.3	0.21–0.45
Cabbage, inner leaves	1	4.4	—
Grass, vacuum dried	3	37.2	28.7–50.2
Yeast, <i>Torula utilis</i> , dried	1	42.6	—
Yeastrel	1	—	—

accuracy of the method decreases with decreasing concentration of riboflavin in the material examined. This low variation was confirmed by Bolton (private communication), who found with our procedure a coefficient of variation of 5.3% when analyzing ground nut cake in 12 repeats (2.38 μ g riboflavin/g, standard deviation ± 0.127 , or 5.3%).

RESULTS

Various foodstuffs were examined for their content of riboflavin. As may be seen in Table 2 the method is applicable to a wide variety of material. A number of foodstuffs have been assayed both chemically and microbiologically. The latter method is described in a following paper (Kodicek, 1949). Good agreement was found.

The method described above obviates the lengthy process of adsorption. The extraction of riboflavin when combined with enzymic digestion seems to be complete. The resulting extracts are clear and almost colourless.

It is important to point out that when dithionite is used as the reducing agent, blue light is

essential for obtaining the correct blank reading. We have observed that under ultraviolet light $\text{Na}_2\text{S}_2\text{O}_4$ reduces very strongly, or rather extinguishes almost completely, the total fluorescence of the extract. When a cell containing this reducing agent is placed in a beam of ultraviolet light in front of a cell containing riboflavin, the solution does not fluoresce. When blue light is used as the exciting source the fluorescence is almost unaltered.

SUMMARY

1. A modification of a fluorimetric method for the estimation of riboflavin has been described.

2. It estimates directly the fluorescence of riboflavin by using a sensitive photocell-galvanometer circuit without recourse to adsorption.

3. Washing of the extracts with chloroform and oxidation by potassium permanganate and hydrogen peroxide reduces the interference by unspecific fluorescent substances. The reduction of riboflavin by sodium dithionite has been used to obtain the value of the blank.

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The Fluorimetric Estimation of Nicotinamide in Biological Materials

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Scudi (1946) has reported that when a solution of nicotinamide is treated with cyanogen bromide (CNBr) and then extracted with isobutanol at an alkaline pH it fluoresces like the F_2 compound formed from N^1 -methylnicotinamide. Pyridoxal, vitamin B_1 , N^1 -methylnicotinamide and certain alkaloids also produce a similar fluorescence under these experimental conditions. Kodicek (1947) has modified the conditions by using methyl ethyl ketone in a single phase at an alkaline pH instead of

extracting with isobutanol. Even then vitamin B_1 , pteroylglutamic acid, N^1 -methylnicotinamide, pyridoxal and quinolinic acid interfere.

On re-examination of the reaction we have found that a fluorescent compound can be obtained from nicotinamide on treatment with cyanogen bromide, and on standing at an alkaline pH, without recourse to an organic solvent. By further modifications it was possible to eliminate the interference of other fluorescent substances. A method has thus been

worked out for the specific estimation of nicotinamide in various materials in the presence of other nicotinic acid derivatives

EXPERIMENTAL

Fluorimeter The photoelectric fluorimeter used in these experiments was of the 'one photocell type', with Wood's and calorex filters fitted between the incident light and the solution, and Wratten no 47 and no 2A filters placed between the solution and the photocell. A sensitive mirror galvanometer (3800 mm / 1 μ amp) was placed at a distance of 2 m from the scale to obtain a high degree of sensitivity.

Reagents NaOH (5N), CNBr, prepared fresh just before the experiment by adding ice cold 10% aqueous NaCN drop by drop from a burette to ice cold saturated bromine water until it is just decolorized, 0.2M KH_2PO_4 NaOH buffer solution, pH 7.2, metaphosphoric acid (25%), freshly prepared, nicotinamide standard containing 2.5 mg/100 ml water, prepared weekly from a stronger nicotinamide solution

are kept in the dark at room temperature for 45 min, after which the readings of fluorescence can be taken

The blank correction For the blank, the solution under goes the same treatment except that the CNBr reagent is not added ('No CNBr' blank). The galvanometer deflexion due to the blank was usually 1 mm when working with mixtures of pure substances

Variation of factors

The conditions of the reaction described above have been studied by varying the individual factors which may affect the formation of the fluorescent compound

(1) Effect of varying concentrations of CNBr and NaOH and of time in alkali on the development of fluorescence

Fig 1 shows the effect of 2 and 4 ml. CNBr and also of 4, 6 and 8 ml 5N NaOH. It will be seen that 4 ml CNBr (added in the first stage) and 8 ml 5N NaOH (added in the second stage) give the best readings. The maximum fluorescence was attained in 30 min and was constant for at

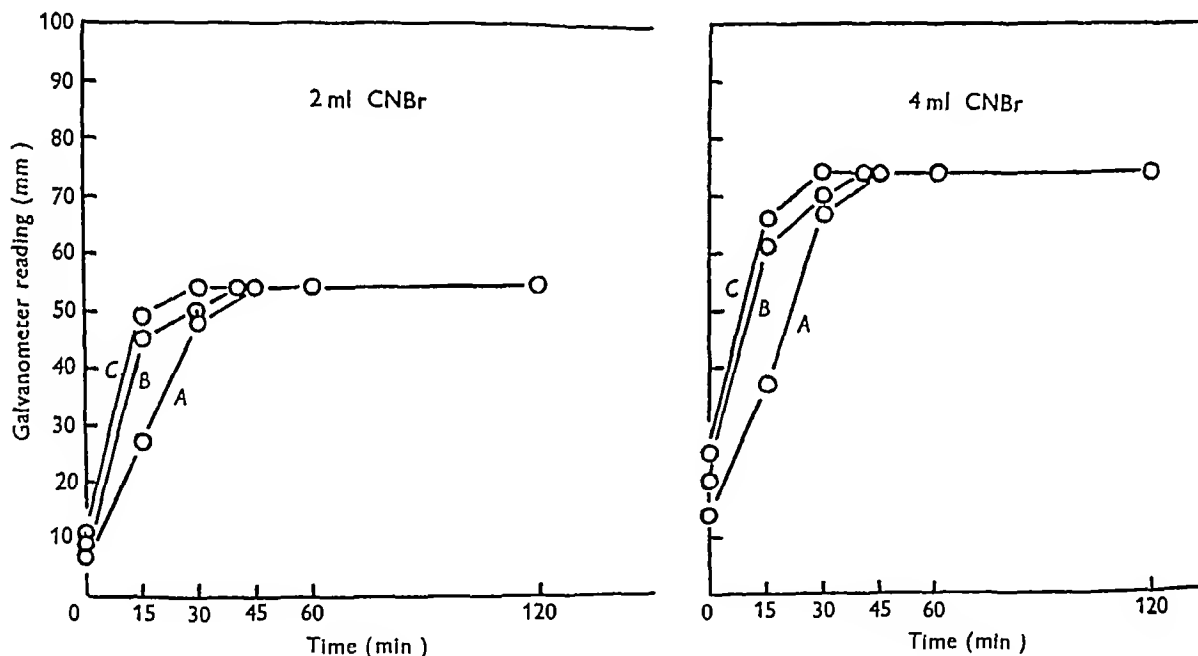


Fig 1 Effects of varying concentrations of CNBr and of NaOH, and of time in alkali on the development of fluorescence of nicotinamide solutions A=4 ml 5N NaOH, B=6 ml 5N NaOH, C=8 ml 5N NaOH

Principle of method for solutions of pure substances

First stage (CNBr treatment) The nicotinamide standard (2 ml containing 50 μ g nicotinamide) and 2 ml. phosphate buffer, are mixed with the CNBr solution and distilled water to about 14 ml in 15 ml glass stoppered measuring flasks. The flasks are immersed for 4 min in a water bath at 56–58°, then cooled in ice cold water for 5 min and the volume made up to 15 ml with water.

Second stage (development of fluorescence) From 5 to 15 ml of the CNBr treated solution are taken, 8 ml 5N-NaOH added, and the volume made up to 30 ml with water in a measuring cylinder. The contents are mixed thoroughly and transferred to a conical flask. The solutions

least another 90 min. A period in the dark of 45 min has therefore been adopted.

Higher concentrations of CNBr (6 ml) caused a decrease of fluorescence. Slightly larger amounts of NaOH (10 and 12 ml.) did not change the maximal fluorescence, but 16 ml caused a diminution. Therefore 4 ml CNBr and 8 ml 5N NaOH have been adopted.

The concentration of the phosphate buffer was found to be of no consequence so long as its final concentration was kept above 0.01M. We used 1 ml 0.2M phosphate buffer in 15 ml of the reaction mixture.

(2) Fluorescence with increasing amounts of nicotinamide

Fig 2 shows the fluorescence obtained with increasing concentrations of nicotinamide. The fluorescence is proportional

to the concentration of nicotinamide up to a final value of about 0.6 $\mu\text{g/ml}$

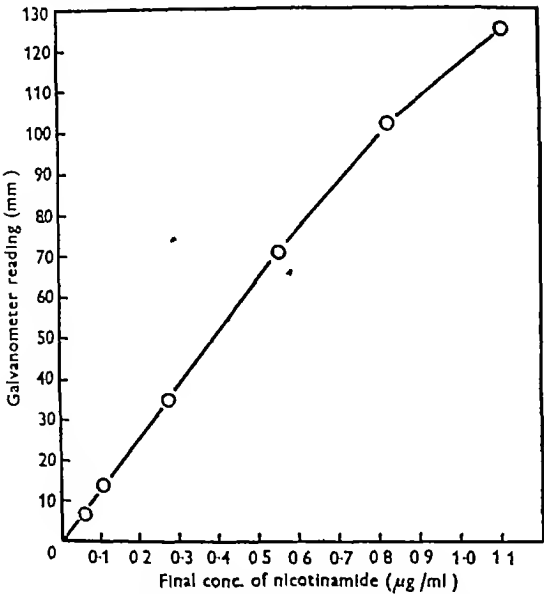


Fig 2 Fluorescence obtained with increasing amounts of nicotinamide

(3) Elimination of interference by other fluorescent compounds

The following compounds were tested and found not to fluoresce: *N*¹-methylnicotinamide, nicotinic acid, coramine, quinolinic acid, α picolinic acid, trigonelline, nipecotic acid (piperidine 3 carboxylic acid), urea, uric acid, yeast nucleic acid, choline chloride, *p* aminobenzoic acid, adenosinetriphosphate, inositol, biotin, calcium pantothenate, riboflavin and indole. On the other hand, vitamin B₁ and diphosphopyridine nucleotide gave a fluorescence under the conditions of our test. Pyridoxal, pteroylglutamic acid and nicotinic thioamide (Karrer & Schukri, 1945) gave a slight fluorescence.

The fluorescence due to vitamin B₁ and coenzyme I was particularly strong. To eliminate this interference, two possible ways were explored: (1) the use of another blank, i.e. the 'No NaOH' blank, and (2) pre-treatment of the vitamin solution, prior to the CNBr treatment, by acidification to remove acid insoluble compounds, like folic acid, followed by weakly alkaline digestion which destroys the vitamin B₁ fluorescence but does not affect that due to nicotinamide.

The 'No NaOH' blank, which was obtained by omitting the addition of 8 ml 5*N* NaOH in the second stage, proved to be unsuitable. On the other hand, the pre-treatment eliminated the interference by other substances, and did not diminish the fluorescence of nicotinamide, present as such or formed from other pyridine compounds. The results are seen in Table 1 which gives galvanometer readings due to the fluorescence of various compounds with and without pre-treatment.

Pre treatment of the solution The solution to be tested is adjusted to pH 2 with conc. HCl and allowed to stand for 5 min at room temperature. If any precipitate is formed, it is centrifuged off. The solution, in a conical flask, is then brought to pH 9.4–9.6 with 20% NaOH (thymol blue as external indicator) and immersed in a boiling water bath for 30 min, after which it is cooled, adjusted to pH 7.2, and made up to a known volume. The steps described under 'Principle of method' are then performed.

From Table 1 it can be seen that this 'pre-treatment' does not affect the intensity of the fluorescence of nicotinamide. Diphosphopyridine nucleotide, when tested in equimolar concentration, gave a fluorescence of the same magnitude as nicotinamide, indicating that the molecule had been split between the pyridine ring and ribose, and free nicotinamide formed. Nicotinic thioamide was converted to a compound giving a fluorescence, but of a lower intensity than that of nicotinamide. As there is no evidence that this compound occurs in nature, this finding is only of theoretical interest. All the other compounds tested by the final procedure showed either no, or little, fluorescence, which in every case was accounted for by the 'No CNBr' blank. Similar

Table 1 Specificity of fluorescence

Substance	Final conc ($\mu\text{g/ml}$)	Galvanometer reading (mm)					
		Without pre treatment			With pre treatment final procedure		
		'No CNBr' blank	'No NaOH' blank	Full fluorescence	'No CNBr' blank	'No NaOH' blank	Full fluorescence
Nicotinamide	0.55	1	1	71	1	1	71
Coenzyme I (48% purity)	6.2*	46	1	70	2.5	1	72
Nicotinic thioamide	1.1	1	1.5	2	1	1.5	45
Aneurin	0.55	5	103	101	1	2	1
Nicotinic acid	1.1	1	1	1	1	1	1
Coramine	1.1	0.5	0.5	0.5	0.5	0.5	0.5
N ¹ Methylnicotinamide chloride	1.1	1	1	1	1	1	1
Quinolinic acid	1.1	1	1	1	1	1	1
α Picolinic acid	1.1	—	—	—	1.5	3	1.5
Trigonelline	1.1	—	—	—	1	1	1
Urea	1.1	—	—	—	1	1	1
Pteroylglutamic acid	1.1	1	21	1	1	16	1
Pyridoxal	1.1	1	46	1.5	2	42	2
Riboflavin	0.78	1	11	1	4	16	4

* Equimolar with nicotinamide

results were also obtained when mixtures of the above substances were tested. The only exception was nicotinic acid, the CNBr addition compound of which decreased the fluorescence of CNBr treated nicotinamide. The nature of this finding was examined in the following experiments

(4) *Effect of different concentrations of nicotinic acid on the fluorescence of nicotinamide*

From Fig 3 it can be seen that there is a proportional decrease of fluorescence with increasing concentration of nicotinic acid. The depression is non competitive, it does not depend on the concentration of nicotinamide, since

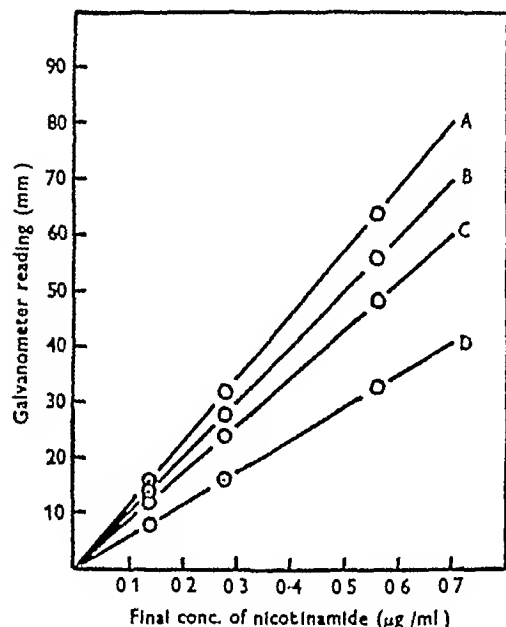


Fig 3 Effect of different concentrations of nicotinic acid on the fluorescence of nicotinamide. A = nicotinamide, B = nicotinamide + 25 μ g nicotinic acid, C = nicotinamide + 50 μ g nicotinic acid, D = nicotinamide + 100 μ g nicotinic acid

when this is varied the same percentage inhibition of fluorescence is obtained, depending on the concentration of nicotinic acid (Table 2). Fortunately, even with large amounts of nicotinic acid, the fluorescence of nicotinamide is proportional to concentration within limits. We shall describe below how the fluorescence of nicotinamide can be quantitatively measured even in the presence of nicotinic acid by using an internal standard of nicotinamide.

Nature of 'quenching' effect of nicotinic acid. The depressing effect of the nicotinic acid CNBr addition compound on the fluorescence of nicotinamide seems to be an 'inner filter' effect. It occurs only with nicotinic acid, no other pyridine derivative gives a similar result. The 'inner filter' effect was obtained only when nicotinic acid was treated with CNBr, irrespective of whether the nicotinic acid was added, as such, at the start of the procedure or added as the CNBr complex immediately before the reading of the fluorescence. Similar results were obtained when a nicotinic acid CNBr solution was placed between the fluorescent nicotinamide solution and the incident ultraviolet light.

Table 2 *Percentage inhibition of fluorescence of nicotinamide by nicotinic acid*

Nicotinic acid (μ g)	Nicotinamide (μ g)	Galvanometer reading (mm)	Inhibition (%)
0	12.5	16	0
0	25	32	0
0	50	64	0
Mean			0
25	12.5	14	12.5
25	25	28.5	11.0
25	50	56.5	11.7
Mean			11.7
50	12.5	12	25
50	25	24	25
50	50	48.5	24.2
Mean			24.7
100	12.5	8	50
100	25	16	50
100	50	32.5	49.3
Mean			49.8

Final procedure for the estimation of Nicotinamide

Extraction and pre treatment

The material to be tested (5 g) is weighed out, cut up finely with scissors if necessary, and ground with sand together with a small amount of 0.1 N HCl (1–2 ml). The ground tissue is transferred with 40 ml water into a 100 ml beaker, and heated in a boiling water bath for 30 min. The solution is cooled, brought to pH 2 with concentrated HCl, centrifuged, and the residue washed with 10 ml 0.1 N HCl and centrifuged. The combined centrifugates are brought to a known volume with 0.1 N HCl (usually 40 ml). Freshly prepared 25% metaphosphoric acid (6 ml) is added. The precipitate is centrifuged after standing for 5–10 min at room temperature. The clear solution is brought to pH 9.4–9.6 (thymol blue as external indicator), and is then heated in a beaker in a boiling water bath for 30 min. The solution is cooled, carefully adjusted to pH 7.2, and the volume made up to 50 ml. It is then filtered through Whatman no. 5 filter paper, and is ready for the next stage of the estimation. An amount of the extract (referred to as U) is taken to contain about 5–25 μ g of nicotinamide.

Development of fluorescence

First stage (CNBr treatment) Three 15 ml glass stoppered measuring flasks are taken (preferably in duplicate), and the reagent mixed according to the following scheme.

Flask A (blank) x ml U + 2 ml 0.2 M phosphate buffer + (12– x) ml water

Flask B (unknown) x ml U + 2 ml 0.2 M phosphate buffer + 4 ml CNBr solution + (8– x) ml water

Flask C (unknown + internal standard) 1 ml (25 μg) nicotinamide standard + x ml *U* + 2 ml 0.2M-phosphate buffer + 4 ml CNBr solution + (7 - x) ml water

The flasks, after their contents have been thoroughly mixed, are immediately placed for 4 min in a water bath at 56–58°. It is convenient to suspend them on the rim of the water bath by a string counterbalanced by the stopper. The solutions are then cooled in ice-cold water for 5 min and the volume made up to 15 ml with water.

Second stage The CNBr-treated solution (5 ml) is placed in a measuring-cylinder (up to 15 ml can be taken, depending on the concentration of nicotinamide and the clearness of the solution). NaOH (8 ml, 5N-) is added, and the volume made up to 30 ml with water. The contents are mixed and transferred to conical flasks which are kept for 45 min at room temperature in the dark. The readings of fluorescence are then taken.

Calculation

Let a = galvanometer reading corresponding to flask A, b = galvanometer reading corresponding to flask B, c = galvanometer reading corresponding to flask C, x = ml of *U* taken for analysis, and f = dilution factor. Then

$$\text{nicotinamide } (\mu\text{g/g}) = \frac{b-a}{c-b} \frac{25}{x} f$$

Reproducibility of results

Replicate estimations of the nicotinamide content of a specimen of rat muscle using the method described here have been statistically analyzed and compared with nicotinic acid results obtained with a modified *p*-aminoacetophenone procedure (Wang & Kodicek, 1943, Harris & Raymond, 1939). It will be seen that the standard error is small. The results are shown in Table 3.

Table 3 *Reproducibility of results*

Rat muscle (sample no.)	Nicotinamide ($\mu\text{g/g}$ tissue)	Total nicotinic acid ($\mu\text{g/g}$ tissue)
1	62.5	71.2
2	61.0	69.6
3	65.8	68.3
4	64.3	68.9
5	69.5	70.8
6	62.5	72.5
Mean	64.3 (± 3.05 , standard error of an individual estimation)	70.2 (± 1.66 , standard error of an individual estimation)
Standard error of the mean	1.24	0.68

RESULTS AND DISCUSSION

The method as described has proved a convenient and rapid procedure for the estimation of nicotinamide. It seems to be reasonably specific, as no naturally occurring substances capable of fluorescence which we have tried so far have been found to interfere. The quenching which is experienced in extracts is corrected for by the internal standard.

The method estimates total nicotinamide either present as such or derived from nicotinamide-containing compounds such as coenzyme I. These break down during the pre-treatment and nicotinamide is liberated. Precautions must be taken which have been already described by Wang & Kodicek (1943) in the CNBr reaction: the pH must be adjusted carefully to 7.2, and only fresh cyanogen bromide, prepared from ice-cold solution, should be used.

Table 4 *Nicotinamide and nicotinic acid content of biological materials*

Material analyzed	Present authors		Krehl <i>et al.</i> (1946)	
	Nicotinamide ($\mu\text{g/g}$)	Total nicotinic acid ($\mu\text{g/g}$)	Nicotinamide ($\mu\text{g/g}$)	Total nicotinic acid ($\mu\text{g/g}$)
Rat				
Liver	125	119	148	154
Muscle	55	52	73	78
Kidney	83	78	79	87
Heart	91	94	—	—
Spleen	40	42.7	—	—
Lung	19	17	—	—
Brain	35	41.8	—	—
Testis	18	21.6	—	—
Wheat bran	0	165	48	245
Wheat flour (100% extraction)	<5	42	15	49.7
Wheat germ	20.8	39.2	20.7	43.5
Dried yeast	185.5	380	—	—
Baker's yeast	100.4	199	—	—
Brewer's yeast	62.8	124.8	—	—
Marmite	0	640	—	—

Table 4 shows the results obtained by our method on various materials. The values were compared with the total nicotinic acid content estimated chemically by a modified *p*-aminoacetophenone procedure. The results of Krehl, Huerga, Elvehjem & Hart (1946) on similar materials have been listed in this table for comparison so far as they have been reported. The method used by Krehl *et al.* is based on a microbiological procedure using *Leuconostoc mesenteroides*, which estimates only free nicotinic acid. The nicotinamide and compounds other than nicotinic acid are therefore calculated by difference between values for the hydrolyzed

and unhydrolyzed sample Our figures agree fairly well with those of the American workers

The rat tissues which we have studied seem to contain almost all their nicotinic acid in the form of amide, bound or free Yeast contains about 50 % of the vitamin as amide compounds From our results we do not know whether the free nicotinic acid found in yeast is an artifact, caused by rapid breakdown of the amide to nicotinic acid, though the same proportion of the acid and amide has been observed both in dried and fresh yeasts Autolyzed yeast and marmite evidently contain only the nicotinic acid and no amide

Cereal products give interesting results Krehl *et al* (1946) reported that a certain proportion of the high amount of nicotinic acid compounds in bran is possibly present as the amide, though they qualify their statement that possibly the precursor of nicotinic acid (Kodicek, 1940, Krehl & Strong, 1944, Krehl, Elvehjem & Strong, 1944) may contribute to the apparent nicotinamide value We did not find any nicotinamide in bran and conclude that the 'precursor' of nicotinic acid present in this cereal must break down on our pre treatment to free nicotinic acid and not to the amide On the other hand, wheat germ contained about 50 % of its vitamin content as nicotinamide The negative results for 100 % extraction whole-wheat flour seem to indicate that only small amounts of nicotinamide are present which would not be detected because of the large experimental error occurring in low-vitamin extracts

SUMMARY

1 A quantitative method for the estimation of nicotinamide has been developed, based on the formation of a fluorescent compound from nicotinamide on treatment with cyanogen bromide under specified conditions

2 The method eliminates possible interference by other compounds giving a similar fluorescence under the conditions of the test by the use of acid and alkaline pre-treatment and of a special blank It estimates the total nicotinamide content, including the free and bound forms

3 The procedure gives specific and reproducible results for biological materials and for cereals, and the results agree well with the reported micro biological values

4 Practically all the vitamin in rat organs and muscles seems to be present in the form of the amide, bound or free In bran, no nicotinamide could be detected before or after digestion It is assumed that the breakdown product of the 'precursor' of nicotinic acid present in bran is the free acid and not the amide Yeast and wheat germ seem to contain about 50 % of the vitamin present in the amide form, bound or free

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The Tryptophanase-tryptophan Reaction

9 THE NATURE, CHARACTERISTICS AND PARTIAL PURIFICATION OF THE TRYPTOPHANASE COMPLEX

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(Received 9 July 1948)

The name tryptophanase was given by Happold & Hoyle (1935) to the enzyme complex of *Escherichia coli* which induces and catalyzes the production of indole from tryptophan by non-viable bacterial preparations with the consumption of five atoms of oxygen (Woods, 1935). The present communication describes the preparation of this complex in the cell free state, and subsequent investigation of the components present by a study of the action of various recognized inhibitors, followed by resolution and identification of the coenzyme factors involved. A preliminary announcement of some of the results has been made (Dawes, Dawson & Happold, 1947a). The investigation of the nature of the tryptophanase complex (Dawes, Dawson & Happold, 1947b, c) was followed by an examination of its characteristics, and attempts at purification were carried out by ammonium sulphate fractionation. Wood, Gunsalus & Umbreit (1947) have already published results of a partial purification of the enzyme effecting fission of the tryptophan molecule using a similar technique to ours, and they have shown independently that pyridoxal phosphate is the coenzyme for the reaction, while they have shown also that the products of the reaction are one mole each of indole, pyruvic acid and ammonia.

METHODS

Washed suspensions of *Esch. coli* were obtained from cultures grown for 18 hr at 37° on tryptic digest of casein solidified with agar. Indole was determined by the method of Happold & Hoyle (1934). For small amounts of indole the rosindole colour was developed with 5 ml Ehrlich's reagent and read in the Hilger Spekker absorptiometer using Ilford 605 spectrum yellow green filters. The curve is linear over the range 1–12 µg indole.

For growth experiments the following amino acid medium was used: L-cystine, 1 mg; glycine, 5 mg; DL-valine, 10 mg; sodium DL-glutamate, 50 mg; sodium lactate, 150 mg; NaCl, 50 mg; Na₂HPO₄ · 12H₂O, 25 mg; KH₂PO₄, 3.5 mg; MgSO₄ · 7H₂O, 3 mg; and FeSO₄ · 7H₂O, 35 µg. Water to 10 ml, pH adjusted to 7.4.

For the microbiological assay of riboflavin the following basal medium was used: 10% acid casein hydrolysate, 200 ml; nicotinic acid, 200 µg; calcium pantothenate, 100 µg; p-aminobenzoic acid, 100 µg; biotin, 2 µg;

pyridoxine, 400 µg; vitamin solution, 2 ml; L-leucine, 20 mg; DL-isoleucine, 20 mg; mineral solution, 4 ml; FeSO₄ · 7H₂O, 800 µg; DL-tryptophan, 100 mg; L-cystine, 800 mg; KH₂PO₄, 4 g; glucose, 20 g; liver eluate fraction, 2 ml. Water to 2 l, pH adjusted to 6.8. The mineral solution contained MgSO₄ · 7H₂O, 22.5 g; CuSO₄ · 5H₂O (1% w/v), 5 ml; ZnSO₄ · 7H₂O (1% w/v), 4 ml; MnCl₂ · 4H₂O (1% w/v), 1.5 ml; conc HCl, 3 ml. Water to 100 ml. The vitamin solution contained pyridoxine HCl, 8 mg; choline HCl, 8 mg; aneurin, 2 mg. Water to 20 ml.

Pyruvate was determined by the method of Friedemann & Haugen (1943) using the toluene extraction technique specific for pyruvic acid. The colours were read in the Spekker photoelectric photometer using Ilford 604 spectrum green filters.

The microdiffusion method of Conway (1933) was used for NH₃ determination. 1 ml 0.01N HCl was placed in the centre compartment of the unit and 1 ml samples of the solution under investigation in the outer compartment. KOH (40% w/v, 0.5 ml) was used to liberate NH₃ and the excess acid was titrated with 0.01N-Ba(OH)₂.

Nitrogen determinations were carried out by the micro-Kjeldahl method. Enzyme solution (1 ml) was digested with 1 ml conc H₂SO₄ with K₂SO₄-CuSO₄ catalyst. NH₃ was distilled into 0.01N acid and titrated with 0.01N alkali.

Manometric experiments with the cell free enzyme were performed with Barcroft differential manometers at 37°. Vessels were set up with contents: control vessel, 4 ml phosphate buffer (pH 7.0) + 1 ml enzyme; reaction vessel, 4 ml phosphate buffer (pH 7.0) + 1 ml enzyme + 250 µg L-tryptophan. With NaOH in the centre wells gas evolution occurred both in the control and reaction vessels, when the NaOH was replaced by HCl, controls showed no gas evolution or uptake, but reaction vessels showed a steady evolution of CO₂. Where the latter was being absorbed gas evolution reached a maximum and then fell off. The finding of gas evolution, which is removed by placing acid in the centre well, is difficult to explain. It is apparently a basic gas, but it cannot be NH₃ or any of the common volatile amines, since these would be absorbed in the reaction fluids at pH 7.0 under the experimental conditions. Proteolysis of the enzyme may be responsible for production of this unidentified gas.

Activities of enzyme preparations during the purification procedure were determined under four conditions, viz. tubes containing 0.5 ml enzyme, 0.5 ml (1% w/v) L-tryptophan, 4 ml phosphate buffer (pH 7.6) to which the following additions were made: (a) nil, (b) 30 µg pyridoxal phosphate, (c) 30 µg riboflavin, (d) 30 µg pyridoxal phosphate + 30 µg riboflavin, were incubated at 37° and the indole content determined after 30 min.

EXPERIMENTAL

Action of various inhibitors on tryptophanase activity

Experiments were conducted with washed-cell suspensions and with the cell-free enzyme to determine the effect of various recognized inhibitors of enzyme action on tryptophanase activity with the object of ascertaining the type of enzyme systems comprising the enzyme complex. The percentage inhibition is given in Table 1 for the washed cell suspensions and in Table 2 for the cell-free enzyme.

Cyanide Potassium cyanide exerts a marked inhibitory effect on tryptophanase activity as measured by inhibition of indole production.

Various inhibitors Semicarbazide, hydrazine, hydroxylamine, sodium azide, mercuric chloride, ferrous sulphate and copper sulphate all inhibited to a greater or less degree. Sodium malonate had no effect.

Table 1 *Effect of various inhibitors on tryptophanase activity of washed bacterial suspensions*

(Phosphate buffer (20 ml, pH 7.4), 1 mg DL tryptophan and 0.5 ml washed cell suspension in 125 ml Erlenmeyer flasks. Inhibitor added to desired concentration. Flasks incubated aerobically at 37° without shaking, indole determined at times given below.)

Inhibitor	Concentration (M × 10 ⁻³)	Time of determination (min)	Percentage inhibition
Cyanide	2	15	100
		45	94
	1	15	93
		45	90
	0.6	15	88
		45	87
	0.5	15	83
		45	80
	0.4	15	83
		45	70
Semicarbazide	10	30	64
	2.5	30	40
	1	30	26
Hydrazine	10	30	100
	2.5	30	100
	1	30	100
Hydroxylamine	10	30	100
	2.5	30	100
	1	30	100
Sodium azide	10	30	50
	2.5	30	20
	1	30	7
Mercuric chloride	10	30	100
	2.5	30	100
	1	30	100
Ferrous sulphate	10	30	23
	1	30	2
Copper sulphate	10	30	92
	1	30	76

British anti-lewisite (BAL) Inhibition was manifest over the range of concentration 0.001–0.1 M.

Table 2 *Effect of various inhibitors on tryptophanase activity of cell-free preparations*

(Phosphate buffer (20 ml, pH 7.4), 1 mg DL tryptophan and 0.5 ml cell free enzyme in 125 ml Erlenmeyer flasks. Inhibitor added to desired concentration. Flasks incubated aerobically at 37° without shaking, indole determined at times given below.)

Inhibitor	Concentration (M × 10 ⁻³)	Time of determination (min)	Percentage inhibition
Potassium cyanide	20	90	100
	2	90	93
	1	90	93
	0.6	90	87
	0.5	90	83
	0.4	90	80
Semicarbazide	10	30	29
	2.5	30	19
	1	30	15
Hydroxylamine	10	30	95
	2.5	30	91
	1	30	86
Hydrazine	10	30	100
	2.5	30	91
	1	30	81
Dimedone	Saturated solution in ethanol	60	22
		120	30
		90	22
		180	52
BAL	100	30	100
		60	100
	50	30	100
		60	100
	10	30	99
		60	77
	5	30	90
		60	31
	1	30	Nil
		60	Nil
Sodium malonate	33	30	Nil

Dimedone It was reported by Baker, Happold & Walker (1946) that dimedone has no effect on the tryptophanase activity of viable cells. Experiments were performed with the cell free enzyme in the same manner as the original experiments so that a true comparison might be obtained. The dimedone was used as a saturated solution in ethanol and similar amounts of ethanol were added to the controls. Erlenmeyer flasks (125 ml) were set up with contents: 20 ml phosphate buffer (pH 7.4), 0.5 mg L tryptophan and 0.5 ml enzyme solution, to which additions were made as follows: (a) none, (b) 0.2 ml ethanol, (c) 0.2 ml dimedone solution. In Table 2 are given results from two such experiments using different enzyme preparations, the percentage inhibition produced by dimedone being

Preparation of cell free tryptophanase

Work on the preparation of the cell free complex was begun with an investigation of the effectiveness of acetone drying and subsequent extraction. This method proved to be efficient (Dawes *et al* 1947*a*).

Preparation of acetone dried *Escherichia coli* Cells grown for 22 hr at 37° on a tryptic digest of casein solidified with agar were harvested, washed and filtered through glass wool. Thick, creamy suspensions obtained in this way were poured into 5 vol ice cold acetone with constant stirring. The flocculated cells were filtered off on a Buchner funnel and washed successively with acetone, acetone ether (1:1 v/v mixture) and finally ether. Drying was achieved by draining on the Buchner and placing in a CaCl_2 desiccator. During the filtration, care must be exercised that the cells are kept under the wash liquids, and not allowed to become dry until the final ether washing has been performed. If this is not observed the preparation obtained is brown and glutinous instead of the usual white powder, and its activity is seriously impaired.

Extraction of tryptophanase from acetone dried cells Extracts were made with buffer solutions of pH 5, 7 and 9 by incubating 20 mg powder/ml buffer for 2 hr at 37°, centrifuging and testing the clear supernatant for tryptophanase activity. After a few trials the incubation period was increased to overnight. The activity of the extracts was measured by the indole production when 1 ml extract was incubated with 5 ml phosphate buffer (pH 7.4) and 1 mg L tryptophan. Activity was nil with extraction at pH 5, slight at 7 and more marked at 9. A more detailed study resulted in the use of borate buffer (pH 8.6) and overnight extraction, and this led to an investigation of the efficiency of salt solutions as extractants, namely KCl and Na_2SO_4 . KCl was shown to be more effective than Na_2SO_4 and far more effective than borate buffer (pH 8.6).

The effectiveness of extraction at varying potassium chloride concentrations, viz saturated solution (approx 27 g/100 ml), 24 g/100 ml (3.2 M), 12 g/100 ml (1.6 M) and 8 g/100 ml (1.06 M) was compared. Of these, maximal extraction was obtained with a concentration of 1.6 M, which is practically a half-saturated solution, and the latter was used in all subsequent work.

The efficiency of the extraction procedure used was determined by relating the activity of the preparation to the dry weight of material used. The activity was expressed as μg indole produced/mg dry wt of material/30 min. Table 5 records the activity at three stages of the procedure, from which it will be seen that the potassium chloride extract contains approximately one third of the activity of the original bacterial suspension, and that the potassium chloride extracts approximately 80% of the activity of the acetone dried cells. About 40% of the original activity is found in the acetone dried cells.

Storage of preparations Acetone dried cells retained activity over several months without appreciable loss even if stored in a covered dish at room temperature without

keeping in a desiccator. KCl extracts could be stored at 0° for a period without serious loss of activity, e.g. over a period of 16 days there was 11% loss of activity. When extracts were stored at 0° it was often found that a precipitate of protein separated out, this was shown to be devoid of tryptophanase activity and could be discarded.

Table 5 Activity of enzyme at stages of extraction of tryptophanase

(Tubes incubated at 37° with 4.5 ml phosphate buffer (pH 7.6), 2 mg L tryptophan and 0.5 ml enzyme preparation.)

Material	Activity	
	(μg indole/mg dry wt/30 min)	Percentage
Cell suspension	1.59	100
Acetone dried cells	0.63	39.6
KCl extract of acetone dried cells	0.54	33.5

Resolution of the cell free enzyme

The cell-free enzyme, obtained as potassium chloride extracts from acetone dried cells, was subjected to 18 hr dialysis in a collodion bag, against distilled water. This reduced the activity but inactivation was incomplete. The still active protein was kept at 0° for 7 days and again dialyzed for 18 hr. The dialysates were concentrated under reduced pressure to small volume, they were yellowish in colour and fluorescent in ultraviolet light. The apo enzyme and dialysate were devoid of tryptophanase activity when tested separately with 0.5 mg L tryptophan in 5 ml phosphate buffer (pH 7.4), but when added together activity was regained, though much reduced. Thus, 1 ml enzyme before dialysis produced 26 μg indole in 60 min, while 1 ml reconstituted enzyme after dialysis produced 3 μg indole in 60 min. This indicates that dialysis causes serious damage to the apoenzyme of tryptophanase. If dialysis is performed against running water then the enzyme can be inactivated after 18 hr dialysis.

Glucose-grown cells Experiments were also performed with potassium chloride extracts of acetone dried *Esch. coli* obtained from 18 hr growth on tryptic digest casein agar to which 1% (w/v) glucose had been added. These cells were devoid of tryptophanase activity (Happold & Hoyle, 1936). The extracts were dialyzed in a manner similar to that described above to yield a protein fraction and a dialysate.

Tubes were set up with the following contents: 5 ml borate buffer (pH 8.6) and 0.5 mg L tryptophan and (a) 1 ml apoenzyme + 1 ml glucose dialysate, (b) 1 ml protein fraction + 1 ml ordinary dialysate. After 60 min incubation, the indole production was (a) 2 μg , (b) nil. From this, and similar experiments, it was evident that the dialysate from glucose grown cells was capable of activating the apoenzyme of

tryptophanase, whereas the protein moiety of glucose grown cells could not be activated

Riboflavin assay Samples of apoenzyme and dialysate, obtained as above, were assayed for riboflavin content by the microbiological method using *Lactobacillus casei* E (Table 6) From the curve for acid production with known amounts of riboflavin it was found that the riboflavin content of 1 ml apoenzyme solution was 0.265 μg at 72 hr and 0.255 μg at 90 hr giving a mean figure of 0.260 μg riboflavin/ml With the dialysate the figures obtained were more divergent, viz 0.135 and 0.095 μg at 72 and 90 hr respectively, giving a mean figure of 0.115 μg riboflavin/ml

Thus by microbiological assay both the apoenzyme and the dialysate of tryptophanase (which were of themselves inactive for indole production) contain riboflavin Apparently some riboflavin, presumably as a prosthetic group, is dialyzed from the protein

Table 6 Assay of protein and dialysate of tryptophanase for riboflavin

(Medium (20 ml.) was dispensed in 125 ml Erlenmeyer flasks and sterilized for 10 min at 10 lb/sq in To each flask were added 0.2 ml CaCl_2 solution (0.5% w/v) and further additions as below Apoenzyme and dialysate used were obtained from KCl extracts and to counteract the effect of added KCl, additions of equivalent amounts of KCl solution were made to control flasks (see Chattaway, Happold & Sandford, 1943) Acid production determined at 72 and 90 hr by titration with 0.1 N NaOH)

Exp	Addition		Acid production (ml 0.1 N)	
			72 hr	90 hr
1	Nil	Nil	Nil	Nil
2	Riboflavin	0.1 μg	0.80	1.55
3	"	0.2 "	1.65	2.85
4	"	0.3 "	2.95	4.60
5	"	0.4 "	4.50	9.60
6	Protein	1.0 ml	2.50	3.70
7	"	0.1 "	0.40	0.60
8	"	0.01 "	Nil	Nil
9	"	0.001 "	Nil	Nil
10	Dialysate	1.0 "	0.95	1.30
11	"	0.1 "	Nil	Nil
12	"	0.01 "	Nil	Nil
13	"	0.001 "	Nil	Nil

Spectrophotometric examination of dialysate The original spectrophotometric analysis was kindly undertaken by Dr F. Bergel The sample had a dry weight of 87 mg/ml and the analysis revealed only one characteristic band with a maximum at 255.5 $m\mu$ and a minimum at 229 $m\mu$ The shape of the curve obtained was similar to that of nicotinic acid which has its maximum at 260–262 $m\mu$

Spectrochemical examination of apoenzyme The apoenzyme obtained by dialysis was examined spectrochemically by Mr J. Ewles to detect and

estimate any metallic elements present A Littrow type spectrograph was used and a total exposure of 250 sec was necessary Iron was detected and the spectrum of the apoenzyme was compared with that of iron solutions of varying strength Iron was present to a concentration of 1.5 parts/1,000,000 Other metallic elements were not detected

Identification of the coenzyme factors

Attempts were made to reactivate the apoenzyme using pyridoxal phosphate, riboflavin and diphosphopyridine nucleotide (DPN), the presence of which were considered as possibilities in view of the interpretation of results obtained with inhibitors and spectrophotometric analysis

A cell-free extract was dialyzed against water for 42 hr and found to be quite inactive Tubes were then set up with contents 5 ml phosphate buffer (pH 7.0) + 1 ml apoenzyme + 1 mg L-tryptophan, to which the following additions were made (a) nil, (b) 30 μg pyridoxal phosphate, (c) 200 μg DPN After 60 min incubation at 37° the indole production was (a) nil, (b) 2 μg , (c) nil

Experiments were repeated with additions as indicated in Table 7 Indole production was negative after 60 min incubation so the experiment was repeated, incubating for 48 hr and plating at the conclusion to make sure that growth had not occurred

Table 7 Action of various coenzyme factors in reactivation of apoenzyme of tryptophanase

(Tubes set up with contents 5 ml phosphate buffer (pH 7.0), 1 ml apoenzyme (prepared by dialysis against water for 42 hr), 1 mg L-tryptophan Additions as below Incubation at 37° PP=30 μg pyridoxal phosphate, R=20 μg riboflavin, DPN=200 μg diphosphopyridine nucleotide)

Additions to 1 ml apoenzyme	Indole (μg) at 48 hr
Nil	Nil
PP	Nil
PP + DPN	185
PP + R	5
R	Nil
R + DPN	286
DPN	160
R + PP + DPN	395

Table 7 shows that reactivation was achieved with the combined addition of pyridoxal phosphate, riboflavin and diphosphopyridine nucleotide In such reactivation experiments where positive and negative findings have been found at different times with any single factor, it is the positive finding which is significant, since with a coenzyme complex the limiting factor is the one which is in short supply In samples of apoenzyme assayed riboflavin was present, but the apoenzyme was quite

inactive for tryptophanase. Here again is evidence that dialysis exerts a profound effect on the apoenzyme presumably accelerating denaturation.

Effect of carrier systems on tryptophanase activity

Verdoperoxidase The absence of a normal cytochrome system in *Esch. coli* (Keilin & Harpley, 1941), coupled with the fact that a study of the action of iron inhibitors indicated that iron catalysis was occurring, led to an investigation of related carrier systems. Verdoperoxidase (VPO), which has been considered as a more or less modified cytochrome *a* derivative, was prepared to investigate its effect on tryptophanase activity. The preparation was carried out according to the method of Agner (1943) from 1 l of pleural fluid obtained from a tuberculous patient, and the resulting solution was tested for its effect on both washed-cell suspensions and the cell-free enzyme.

For these experiments 125 ml Erlenmeyer flasks were set up with 10 ml phosphate buffer (pH 7.4), 0.5 mg L-tryptophan, 0.5 ml VPO solution and either 0.5 ml bacterial suspension or 1 ml cell-free enzyme. Controls contained no VPO. The results in Table 8 show that VPO stimulated indole production in both cases.

Table 8 *Effect of verdoperoxidase (VPO) on indole production by washed suspensions of Esch. coli and by cell free tryptophanase*

(125 ml Erlenmeyer flasks contained 10 ml phosphate buffer (pH 7.4), 0.5 mg L-tryptophan and 0.5 ml washed cell suspension or cell free enzyme. Incubation at 37°)

Experiment	Time (min)	Indole production (μg)	
		Control	VPO (0.5 ml)
Washed cell suspension	15	133.2	148.2
	30	200.0	210.6
Cell free enzyme	30	10.5	15.0
	75	26.0	38.0

Cytochrome c Since the results obtained with inhibitors suggested the operation of a cytochrome type of system in the tryptophanase complex, the effect of added cytochrome *c* was investigated. The solution used contained 1 mg cytochrome *c*/100 ml 0.1 M-magnesium chloride solution. Tubes were incubated at 37° with 2.5 ml phosphate buffer (pH 7.4), 2 mg L-tryptophan, 0.5 ml enzyme, to which, in the case of the reaction tubes, 1 ml cytochrome *c* solution (10 μg) was added.

As will be seen from Table 9, cytochrome *c* markedly stimulated indole production.

Table 9 *Effect of cytochrome c on indole production by tryptophanase*

(Tubes incubated at 37° with 2.5 ml. phosphate buffer (pH 7.4), 2 mg L-tryptophan and 0.5 ml. cell free enzyme, 10 μg cytochrome *c* added in reaction tubes)

Time (min)	Indole produced (μg)	
	Control	Cytochrome c
10	62.0	72.9
20	91.5	129.9

Absence of catalase and hydrogen peroxide The cell-free enzyme was devoid of catalase activity as shown by hydrogen peroxide and permanganate titration, and no trace of hydrogen peroxide could be detected when tryptophanase was incubated with tryptophan.

Effect of ATP ATP is without effect on indole production by the cell-free enzyme.

Manometric studies with cell-free tryptophanase

Potassium chloride extracts of acetone dried cell preparations were used for the initial manometric investigations. Results are recorded in Tables 10 and 11, where the effects of sodium hydroxide and

Table 10 *Gas evolution by cell-free tryptophanase on L tryptophan substrate*

(Control vessels 4 ml phosphate buffer (pH 7.0) + 1 ml enzyme. Reaction vessels as above + 250 μg L-tryptophan. NaOH in centre wells for CO₂ absorption. Gas evolution calculated on oxygen constant of the apparatus)

Time (min)	Gas evolution (μl)		
	Control	250 μg L-tryptophan	
		(a)*	(b)*
5	5.9	14.4	16.0
15	7.4	21.6	17.4
45	5.9	27.5	24.8
70	4.5	30.3	36.4
90	4.5	25.9	42.3

* Duplicate experiments run simultaneously

Table 11 *Gas evolution by cell free tryptophanase on L tryptophan substrate, comparison of effect of NaOH and HCl in centre wells*

(Control vessels 4 ml phosphate buffer (pH 7.0) + 1 ml enzyme. Reaction vessels as above + 250 μg L-tryptophan)

Time (min)	Gas evolution (μl)			
	Control		250 μg L tryptophan	
	HCl	NaOH	HCl	NaOH
10	—	10.4	1.46	17.2
20	—	10.4	4.38	17.2
40	—	17.8	14.6	24.1
50	—	17.8	19.0	24.1
65	—	17.8	29.2	25.6
80	—	19.3	40.9	18.4
100	—	19.3	57.0	19.0

Table 12 *Manometric study of tryptophanase preparation from growth supernatants, NaOH and HCl in centre wells*

(Control vessels 3 ml phosphate buffer (pH 7.0) + 2 ml enzyme Reaction vessels as above + 250 μ g L-tryptophan Oxygen uptake is an arbitrary rate obtained from the fall in gas evolution (preceding column))

Time (min)	Gas evolution (μ l)				
	Control		250 μ g L tryptophan		
	HCl	NaOH	HCl	NaOH	Oxygen uptake
5	—	19.3	33.6	41.6	—
15	—	14.9	38.0	35.8	9.8
20	—	13.4	40.9	36.0	11.1
30	—	17.8	42.4	28.7	14.0
40	—	22.3	45.3	20.5	18.5
50	—	25.6	48.2	16.4	18.5
60	—	23.8	49.6	12.4	24.3
70	—	23.8	54.0	8.1	28.6
90	—	23.8	61.4	- 2.0	39.7

Final manometer reading at 6 hr gave oxygen consumption of 71.5 μ l (theoretical 68.5 μ l for complete oxidation of 250 μ g L-tryptophan to indole, CO₂, NH₃ and H₂O)

hydrochloric acid in the centre wells are compared, indicating the evolution of a basic gas by the enzyme control Table 12 records results obtained with a cell-free enzyme prepared from supernatants of *Esch coli* cultures after 22 hr growth on tryptic digest casein agar. The supernatant was treated with half saturated ammonium sulphate at pH 4.8, the precipitate suspended in water and sulphate ions removed by the addition of barium chloride. This preparation produced 15 μ g indole/2 ml enzyme in 30 min.

Manometric work on partially purified enzyme preparations, and also with certain cell-free extracts, led to the discovery that indole can be produced without oxygen utilization, a finding which has also been noted by Wood *et al* (1947). There was no gas evolution either with such preparations. Where indole production was obtained without oxygen uptake the contents of the manometer cups were concentrated and used for paper chromatography. Alanine was not detected in these experiments (see Dawes, Dawson & Happold, 1947d). Wood *et al* (1947) have shown that tryptophanase, purified according to their scheme, contains no alanine or serine deaminase. With our further purified enzyme preparations, which required no oxygen for indole production, alanine likewise was not deaminated.

The products of tryptophanase action on tryptophan

Previously, we believed that the initial product of the fission of the tryptophan molecule was alanine (Dawes *et al* 1947d), which was then oxidized, through pyruvic acid, to carbon dioxide, ammonia and water. Wood *et al* (1947), however, reported that the products of the reaction were one mole each of indole, pyruvic acid and ammonia and that the

enzyme effecting this would not deaminate alanine. In the light of this work we have examined the products obtained by the action of our enzyme preparations for pyruvate and ammonia (Table 13).

Table 13 *Products of tryptophanase reaction on tryptophan*

(Tubes contained (a) 1.3 ml phosphate buffer (pH 7.6), 0.2 ml (2 mg) L-tryptophan and 0.5 ml enzyme, (b) 1.5 ml phosphate buffer (pH 7.6) and 0.5 ml enzyme. After 10 min incubation at 37°, 3 ml 10% (w/v) trichloroacetic acid were pipetted into each tube and the protein precipitate filtered off. Total contents of tubes (5 ml) used for indole determinations, 3 ml samples of control and reaction liquids used for pyruvate determinations and 1 ml samples for ammonia determinations, the control liquid (enzyme only) being used as blank for the Conway (1933) procedure.)

Time of determination (min)	Pyruvate		Indole		Ammonia	
	μ g	μ mol	μ g	μ mol	μ g	μ mol
5	27.5	0.31	37.5	0.32	125.0	7.35
15	49.5	0.56	30.8	0.26	25.0	1.47
10	62.9	0.72	78.5	0.67	21.25	1.25
60	88.0	1.0	22.9	0.20	36.7	2.16

Further experiments in which indole and ammonia determinations only were carried out

10	—	—	93.3	0.79	122.1	7.2
10	—	—	63.0	0.54	83.3	4.9
10	—	—	81.0	0.69	56.1	3.3
10	—	—	95.0	0.81	53.6	3.2
15	—	—	33.8	0.29	64.6	4.6

It will be noticed that while there was reasonable agreement between the pyruvate and indole figures the ammonia was always found in excess. To obtain more figures on this, further experiments were conducted in which indole and ammonia only were

determined, and, as Table 13 shows, ammonia was invariably found in great excess indicating that deamination is preceding fission into indole and pyruvate. Since indolylpyruvic acid does not give rise to indole this would mean that a theoretical yield of indole could not be obtained, an experimentally verified fact. It should be noted, however, that Wood *et al.* (1947) report the production of ammonia in step with indole and pyruvate formation.

Purification of tryptophanase

The following method of purification has been carried out on cell-free extracts obtained from acetone dried cells. The solutions were maintained at pH 7 throughout and stored at 0°. The activity at each stage of the purification was related to the nitrogen content, our arbitrary unit of activity being the amount of indole (μg) produced/mg N/30 min (see Table 14).

The extract obtained from 5 g acetone dried cells (250 ml) was brought to 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ in the ice chest, the precipitate centrifuged down, taken up in 40 ml phosphate buffer and further fractionated by half and full saturation with $(\text{NH}_4)_2\text{SO}_4$. The latter deposit, taken up in 20 ml distilled water, was the most active sample of enzyme obtained, but its activity was rapidly lost on storage. The supernatant from the initial 50% saturation was then fully saturated and the deposit taken up in 150 ml distilled water. This active deposit was now further fractionated by 50 and 70% saturation, leaving the supernatant inactive.

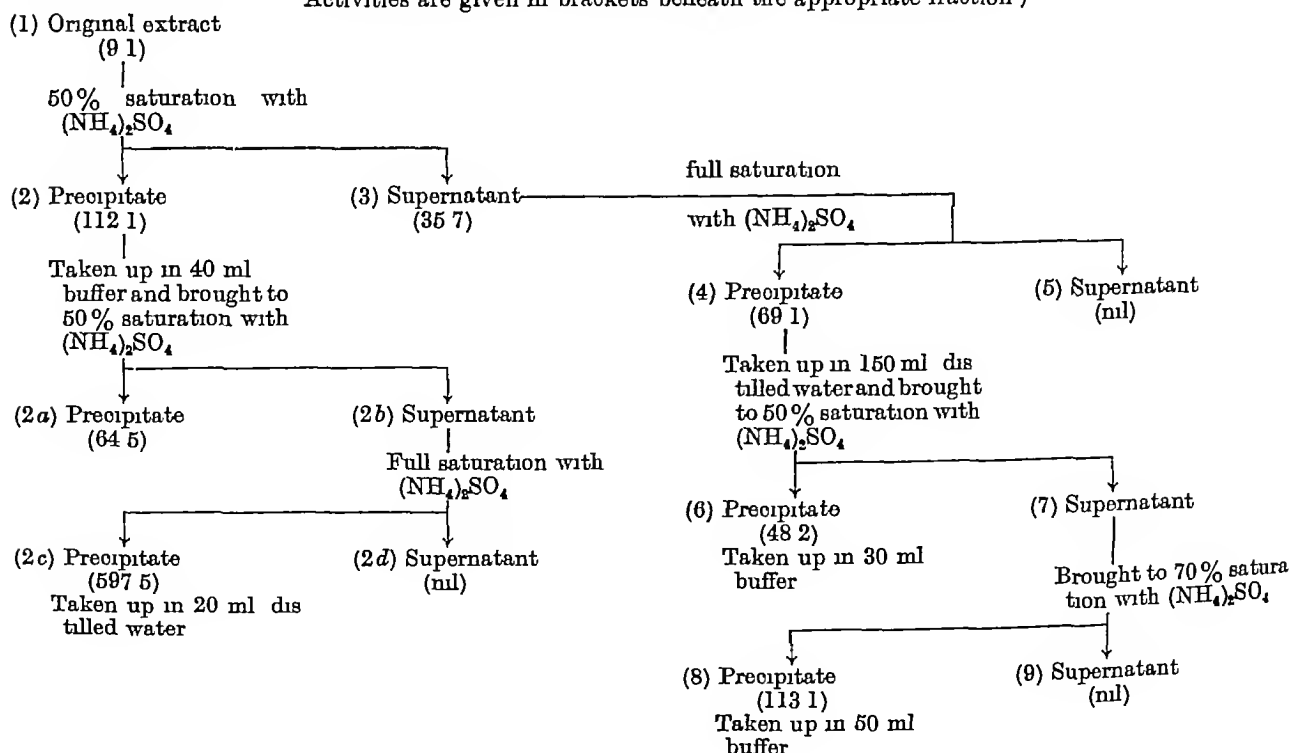
The precipitate from 70% saturation, taken up in 50 ml phosphate buffer, was purified 12 fold and remained active on storage in the ice chest. It was found that the enzyme was completely resolved in samples 2a and 6 *et seq.* and that at this, and subsequent stages, only pyridoxal phosphate was capable of activating the protein moiety. Riboflavin was found to have no activation effect, and, to ascertain whether the protein still contained a riboflavin prosthetic group, sample 8 was examined in ultraviolet light both intact and after hydrolysis, by microbiological assay. Riboflavin was absent. Diphosphopyridine nucleotide (DPN) alone did not reactivate the resolved enzyme, nor did it stimulate indole production in the presence of pyridoxal phosphate or pyridoxal phosphate plus riboflavin. Since this might be due to some DPN being bound to the enzyme protein, the possibility was investigated by an assay for DPN using *Haemophilus parainfluenzae* as the test organism by the method described by Lwoff & Lwoff (1937a). A sample of the resolved, partially purified, enzyme was Seitz filtered and 0.5 ml added to the tubes, 2 ml of the same solution were autoclaved with 2 ml 2N H_2SO_4 for 20 min at 15 lb/sq in, the pH adjusted to 7.6 and 0.5 ml added to other tubes. These were assayed together with controls and tubes containing DPN. Growth occurred only in the tube containing added DPN, and therefore no DPN was present in the enzyme protein. The DPN used in this work was prepared from baker's yeast by the method of Sumner, Krishnan & Sisler (1947).

Sample 2c very rapidly lost its activity on storage, but sample 8 remained active.

From these results, therefore, it would appear that pyridoxal phosphate is the coenzyme which effects fission of the tryptophan molecule.

Table 14 Scheme used for the purification of tryptophanase

(Unit of activity = μg indole/mg nitrogen/30 min with 0.5 ml enzyme in presence of 30 μg pyridoxal phosphate. Activities are given in brackets beneath the appropriate fraction.)



Properties of tryptophanase

Effect of pH Fig 1 shows the variation of activity with the reaction pH. The optimum occurs

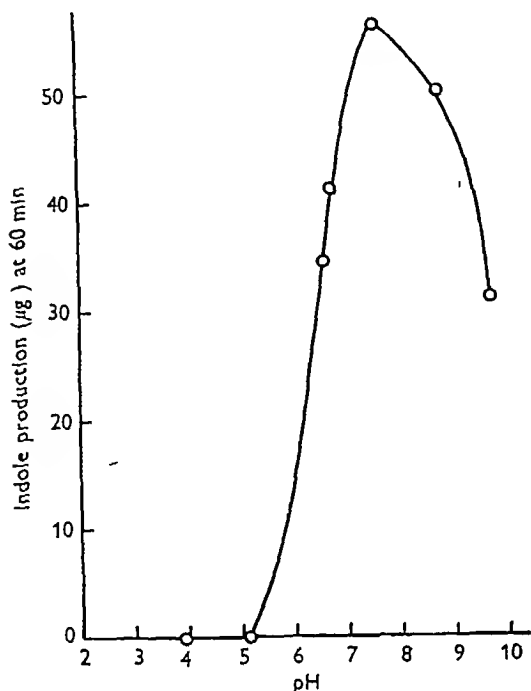


Fig 1 Effect of pH on tryptophanase activity

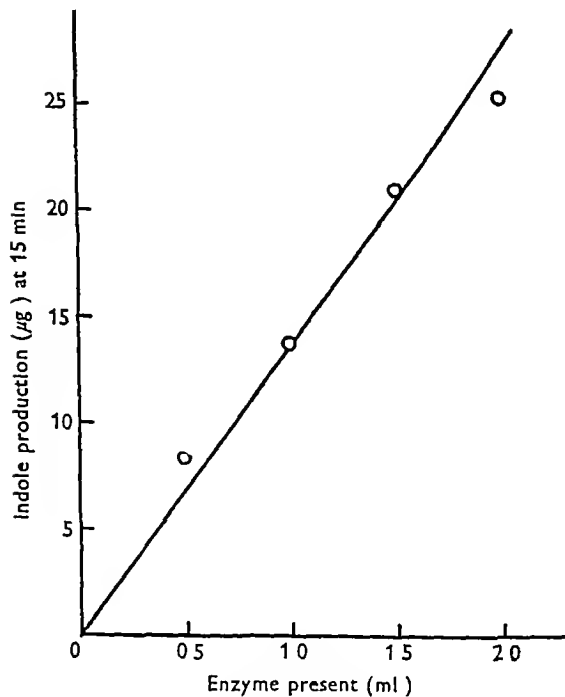


Fig 2 Effect of enzyme concentration on reaction velocity

at pH 7.5. In the original work of Happold & Hoyle (1935), using chloroform-killed *Esch coli*, the optimum was claimed to be pH 8.5, but this was

later acknowledged to be too high, and Gale & Epps (1942) found the optimum pH to be 7.5 with cells grown at pH 7.0. Thus the optimum pH of the cell-free enzyme is identical with that of the intact organism.

Effect of enzyme concentration A linear relationship exists between the enzyme concentration and the velocity of the reaction as shown in Fig 2.

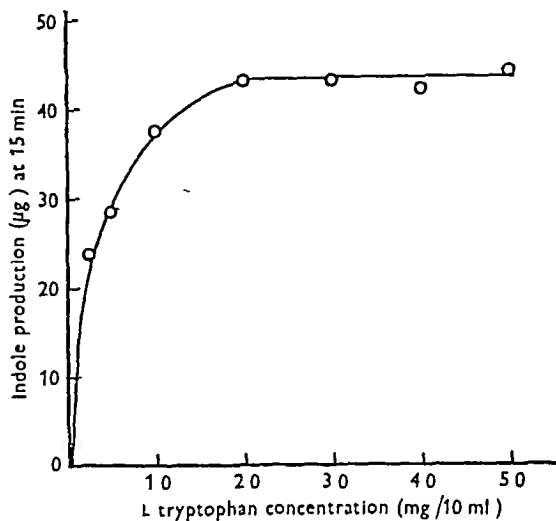


Fig 3 Effect of substrate concentration on reaction velocity

Effect of substrate concentration Fig 3 shows the variation of reaction velocity with substrate concentration. The Michaelis constant of the enzyme was evaluated by the graphical method of Lineweaver & Burk (1934) and the average value obtained was 4.1×10^{-4} moles/l. Wood *et al* (1947) give the value of 2.5×10^{-5} .

DISCUSSION

The tryptophanase complex of *Esch coli* may be obtained as an active cell-free preparation by acetone-drying thick cell suspensions, and extraction of the cell powder with half-saturated potassium chloride solution. Extracts obtained in this way showed good activity, and could be kept in the ice chest for some time without deterioration. The superiority of potassium chloride as extracting agent over other salts tried is presumably due to increased solubility of the enzyme protein in this solution.

Enzyme extracts have been inactivated by dialysis against distilled water, and the activity regained by the addition of a concentrated dialysate to the inactive protein moiety, although the activity displayed by such reactivated systems is greatly reduced in comparison with the original enzyme, probably by denaturation of the enzyme protein. It has been shown that the dialysate

obtained from glucose-grown cells (which are devoid of tryptophanase activity) is also capable of activating the apoenzyme. The protein moiety of glucose-grown cells, however, cannot be activated. This indicates that the essential difference between cells active for tryptophanase and those grown on glucose-agar lies in the protein moiety and that glucose-grown cells possess a full coenzyme for the apoenzyme.

A study of the effect of various inhibitors on tryptophanase activity provided strong presumptive evidence on the nature of components present in the enzyme complex. It was early recognized that cyanide is a powerful inhibitor of tryptophanase and this indicated one or both of the following possibilities: an iron-containing system such as cytochrome, or a carbonyl-containing component such as pyridoxal phosphate with which cyanohydrin formation can occur.

Interference with a component containing the carbonyl group appeared more probable following study of the action of other characteristic carbonyl reagents (semicarbazide, hydrazine and hydroxylamine). With washed cell suspensions the latter two produced complete inhibition down to 0.001M concentration, semicarbazide not being quite so effective. With the cell-free enzyme essentially similar results were obtained. In this respect the action of dimedone is of considerable interest. Baker *et al.* (1946) showed that it had no effect on indole production by washed-cell suspensions, but, using the cell-free enzyme, inhibition was manifest thus bringing dimedone into line with the other carbonyl reagents. This is rather difficult to explain, for it cannot be postulated that the permeability of the bacterial cell to dimedone is the deciding factor. Dimedone, however, is sparingly soluble compared with the other carbonyl reagents employed, and it may be that with the bacterial cells other aldehyde products of bacterial metabolism, such as phosphoglyceraldehyde, acetaldehyde, etc. from carbohydrate breakdown, react preferentially to the exclusion of the coenzyme factor.

The presence of an iron-containing system received support from the action of sodium azide, hydrogen sulphide and carbon monoxide. Keilin & Harpley (1941) showed that *Esch. coli* does not contain a normal cytochrome system. Cytochrome *c* and cytochrome oxidase are absent from the organism and bands *b* and *c* of normal cytochrome are replaced by one band which lies at 560 m μ and which represents an α -band of non-autoxidizable haemochromogen component *b*₁. In this respect the action of verdogeropoxidase, which was found to stimulate indole production with both viable cells and the cell-free enzyme, warrants attention. It has been suggested by Keilin (1933) and Kuhn (1934) that verdogeropoxidase might be considered as a more

or less modified cytochrome *a* derivative and hence the stimulation of indole production by this preparation is of significance.

BAL (2,3-dimercaptopropanol) is an inhibitor of metal-containing enzymes, as shown by Webb & van Heyningen (1947), and since this reagent inhibits tryptophanase it is compatible with the presence of an iron-containing system in the complex. The fact that added cytochrome *c* stimulated indole production with the cell-free enzyme would appear to be explicable in terms of removal of an overall 'bottleneck' in the carrier system of the enzyme. The presence of iron in the apoenzyme was proved by spectrochemical analysis.

The mepacrine inhibition of tryptophanase activity, manifest only with the intact cell and reversed by riboflavin (Dawson, 1946), may be taken as some indication of the functioning of flavoprotein (Haas, 1944; Hellerman, Lindsay & Bovarnick, 1946).

Sulphanilamide inhibits the growth and respiration of *Esch. coli*, but not in the presence of methylene blue, as was shown by Clifton & Loevinger (1942). The bacteriostasis observed in our experiments was in accordance with previous findings, and, in the absence of growth, the non-production of indole from tryptophan is a natural consequence of the lack of metabolic processes. Where the bacteriostasis was overcome, and growth eventually occurred, indole production was found to be normal. It thus appeared that sulphanilamide exerted no damaging effect on the tryptophanase system of the organism, and this was borne out by experiments which demonstrated that sulphanilamide was without effect on the prepared enzyme.

Sodium malonate was reported by Cedrangolo & Filomeni (1943) to inhibit the deamination of natural amino acids by rat liver homogenate, but it was without effect on indole production by tryptophanase and also without effect on ophio-L-amino acid oxidase when tested manometrically by the Warburg technique.

Happold & Waters (1944) found that clavatin was a powerful inhibitor of tryptophanase, but from a wide survey of its action on respiratory enzymes in general, no concrete conclusion as to its mode of action could be reached and, therefore, its action provided no insight into the nature of the enzyme complex.

The cell-free enzyme was shown to be devoid of catalase activity, and to contain no ascorbic acid oxidase or polyphenol oxidase, while Happold (1930) demonstrated that *Esch. coli* contains no catecholase and is incapable of oxidizing dimethyl *p*-phenylenediamine.

The dialysate obtained from a tryptophanase extract was examined spectrophotometrically and

the absorption curve obtained corresponded most closely to that of nicotinic acid. This was evidence for the presence of a nicotinic acid adenine nucleotide. Spectrochemical analysis of a sample of apoenzyme revealed that iron was present, but no other metal was detected.

Reactivation of the apoenzyme obtained by dialysis was achieved with pyridoxal phosphate, riboflavin and diphosphopyridine nucleotide. The presence of riboflavin had been suspected in the earliest work with mepacrine and washed-cell suspensions. It was confirmed by microbiological assay using *Lb casei* E and shown to reside in both dialysate and apoenzyme. It is, apparently, present as prosthetic group, and there is some loss on dialysis.

When the further purification of the enzyme was studied the apoenzyme was apparently free from a flavin prosthetic group as evidenced by ultraviolet fluorimetry and microbiological assay. The flavoprotein present in the cruder preparations is presumably only concerned with the further oxidation of the fission product, but as such it obviously can affect the overall reaction rate in the cell by mass action effect.

Manometric investigation of the cell-free enzyme showed that crude extracts were capable of oxidizing one mole of tryptophan to indole, carbon dioxide, ammonia and water with the consumption of five atoms of oxygen, but on further purification the enzyme produced indole without concurrent oxygen utilization. In several experiments with crude extracts an evolution of gas was recorded with both reaction manometers and control vessels. This could be prevented in the case of controls by placing hydrochloric acid in the centre well instead of caustic soda. This, presumably, meant that a basic gas was being evolved, it is inconceivable that ammonia or one of the volatile amines could produce this effect on account of their great solubility. We place these findings on record, but are unable to offer an explanation of their significance.

Until the work of Wood *et al* (1947) showed that the products of tryptophanase action are one mole each of indole, pyruvic acid and ammonia, and that the partially purified enzyme will not deaminate alanine, we had believed that alanine, which was produced concurrently with indole in mepacrine inhibited systems, was the product of the primary fission. Accordingly we have examined the products of the reaction for indole, pyruvic acid and ammonia, and, while demonstrating the production of indole and pyruvic acid in equimolar amounts, we have invariably found ammonia production to be in excess. This may have some bearing on the results obtained in manometric experiments, where evolution of a basic gas was found, and may be due to production of volatile amines from the enzyme

protein, these remaining in solution while the protein is removed by trichloroacetic acid prior to the ammonia determination, and then being liberated by the alkali treatment of the Conway (1933) procedure. It should be borne in mind, however, that the manometric experiments were done with crude enzyme preparations while the ammonia determinations were on partially purified preparations which, when examined manometrically, showed no oxygen utilization or gas evolution. If the figures obtained represent ammonia formation, then we conclude that indolylpyruvic acid must be formed, since this compound is not degraded to indole by the enzyme it would be an end product. The theoretical yield of indole would, therefore, not be achieved, this has been found experimentally. It should be noted that the accumulation of indole has also an inhibitory effect on the reaction (Fildes, 1938).

Extracts of tryptophanase were purified by fractionation with ammonium sulphate. Activity was found in both half and fully saturated fractions. Fractionation of the former yielded the most active (60-fold increase) and the most labile preparation. It lost activity completely after 3 days. Fractionation of the deposit from full saturation yielded a reasonably stable preparation with a 12-fold increase in activity. From this it seems that the enzyme is readily denatured. This appears to account for the comparatively small degree of purification achieved in the fractionation procedure.

The enzyme was completely resolved by the ammonium sulphate fractionation, and could only be reactivated by the addition of pyridoxal phosphate. At this stage, as noted by Wood *et al* (1947), the enzyme would not deaminate alanine, and produced indole from tryptophan without oxygen utilization, pyruvic acid being one of the end products of the reaction. This treatment, therefore, removes the portion of the tryptophanase complex which effects the oxidation of pyruvic acid. The failure of riboflavin to activate the apoenzyme led us to examine the apoenzyme for the presence of a riboflavin prosthetic group. This was not found by ultraviolet examination or by microbiological assay. Diphosphopyridine nucleotide (DPN) also failed to reactivate the resolved enzyme, neither did it stimulate indole production in the presence of pyridoxal phosphate alone, or together with riboflavin. Although DPN is usually considered as readily dialyzable from the apoenzyme, a sample of the resolved enzyme was assayed for DPN intact and hydrolyzed. None was found. Lwoff & Lwoff (1937b) made the suggestion that triphosphopyridine nucleotide could be present in *Haemophilus parainfluenzae* in combined or free form.

From these findings we conclude that pyridoxal phosphate is the sole coenzyme to participate in

fission of the tryptophan molecule, and to do this it presumably acts as a hydrogen acceptor by reversible aldehyde alcohol interconversion

The enzyme effecting fission has optimal activity at pH 7.5, and a linear relationship exists between the reaction velocity and the enzyme concentration. The Michaelis constant is $4.1 \times 10^{-4} M$. Wood *et al* (1947) give a value of 2.5×10^{-5} for this constant, but state that half maximum rate of reaction is obtained with 35 μg tryptophan/ml. This, when calculated, gives a value of $K_M = 1.71 \times 10^{-4}$ and is more in accord with our own figure.

In conclusion, attention is drawn to the difference between the crude cell-free tryptophanase complex and the purified component which effects fission into indole, pyruvic acid and ammonia. The former carries through also the oxidation of pyruvic acid to carbon dioxide and water. The fact that the activity of preparations, which contain the pyruvic oxidase, is enhanced by riboflavin and DPN suggests that the production of indole is affected by mass action considerations.

Wood *et al* (1947), who reported the function of pyridoxal phosphate as coenzyme factor, used the term 'tryptophanase' for the enzyme effecting the production of indole, pyruvic acid and ammonia without oxygen uptake, in contradistinction to the original use by Happold & Hoyle (1935) to connote the complex which performs the complete oxidation to indole, carbon dioxide, ammonia and water with the consumption of five atoms of oxygen. The fact that the partially purified enzyme, which produces indole, will not deaminate alanine invalidates our earlier conclusion (Dawes *et al* 1947*d*) that alanine is a normal fission product of tryptophan, and means that in the presence of mepacrine a modification of the reaction occurs. Since experiments were performed with washed cell suspensions it is possible that alanine was produced from the pyruvic acid by transamination.

SUMMARY

1 The tryptophanase complex of *Escherichia coli* may be obtained in the cell-free condition by extraction of acetone dried cells with half-saturated potassium chloride solutions.

2 Tryptophanase is inhibited by iron inhibitors, carbonyl inhibitors and BAL. Mepacrine and benzamide, which have inhibitory actions on the tryptophanase activity of viable cells, have little effect on the cell-free enzyme.

3 Tryptophanase activity is stimulated by verdogeroxidase and cytochrome *c*.

4 The enzyme complex has been resolved and shown to require pyridoxal phosphate, riboflavin and diphosphopyridine nucleotide as coenzyme factors.

5 A partial purification of the tryptophanase complex has been achieved by ammonium sulphate fractionation.

6 The purified fraction effects the fission to indole, pyruvic acid and ammonia with pyridoxal phosphate as sole coenzyme under anaerobic conditions.

7 The resolved enzyme does not contain a riboflavin prosthetic group or diphosphopyridine nucleotide and these coenzymes, therefore, appear to be concerned with the further oxidation of pyruvic acid.

8 The fractionation removes the enzyme or enzymes effecting oxidation of pyruvic acid.

We should like to express our gratitude to Prof R. A. Peters for a gift of BAL, Dr E. F. Gale and Prof I. C. Gunsalus for gifts of pyridoxal phosphate and Dr E. A. Zeller for a gift of ophio L-amino acid oxidase. In addition we wish to thank Dr F. Bergel and Mr J. Ewles for spectrophotometric and spectrochemical analyses respectively, Dr J. Ellison for the riboflavin assay, Mr L. Davis for micro-Kjeldahl determinations and Miss M. Horrabin for assistance in the preparation of DPN.

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Metabolism of Polycyclic Compounds

5 FORMATION OF 1 2-DIHYDROXY-1 2-DIHYDRONAPHTHALENES

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Early work on the metabolism of aromatic hydrocarbons indicated that they were converted into phenols (e.g. Schultzen & Naunyn, 1867). Later investigations on the fate of anthracene in the body (Boyland & Levi, 1935) showed that such hydrocarbons may be first converted to diols or dihydroxy-dihydro derivatives, which may decompose to give phenols. Analogous diols have been isolated and characterized from the urine of animals dosed with naphthalene. A laevorotatory form of 1 2-dihydroxy-1 2-dihydronaphthalene was isolated from rat urine by Young (1947) and its structure elucidated. A corresponding dextrorotatory form has now been isolated from rabbit urine and an optically inactive form from the urines of both rats and rabbits. The possible relationship of metabolism of hydrocarbons to carcinogenesis has been discussed elsewhere (Boyland, 1948).

EXPERIMENTAL

Animals were given intraperitoneal injections of a 20% (w/v) solution of naphthalene in arachis oil (2.5 ml/kg body wt) twice weekly and kept in metabolism cages of the Paine type. Urine was collected daily and a small amount of urease added to decompose urea. The pooled urine of each species was continuously extracted with ether for 2-3 days.

Rabbit. Isolation of optically inactive and dextrorotatory forms of 1 2-dihydroxy-1 2-dihydronaphthalene. On evaporation of the ether a small amount of dark-brown solid material was obtained which after several recrystallizations from benzene yielded silky, white needles, m.p. 101°, $[\alpha]_D^{25}$ 0° (c, 1% in ethanol) (Found C, 73.8, H, 6.2. Calc. for $C_{10}H_{10}O_2$ C, 74.1, H, 6.2%) (Cf. Booth & Boyland, 1947). This compound was very soluble in cold ethanol, acetone and ether, and soluble in water to the extent of about 2 g/l. It was only slightly soluble in cold benzene, but readily dissolved on warming and was sparingly soluble in boiling light petroleum.

When the crude, dark brown material obtained by the evaporation of the ether extracts was dissolved in 95% ethanol and continuously extracted with light petroleum (b.p. 40-60°) the first crystals to appear in the light petroleum were rectangular plates. The extraction was interrupted and this material isolated. On continuing the extraction a mixture of this material (which was dextrorotatory) and the optically inactive form was obtained and finally a fraction with no optical activity. The first light petroleum fraction was evaporated and the residual solid recrystallized from benzene giving colourless plates, m.p. 125°, $[\alpha]_D^{22}$ +159° (c, 1% in ethanol) (Found C, 74.0, H, 6.4. $C_{10}H_{10}O_2$ requires C, 74.1, H, 6.2%). The optical activity remained unchanged on recrystallization. This compound was less soluble in benzene and light petroleum than the optically inactive form, and was identical in all physical properties except optical rotation with the compound isolated by Young (1947) from rat urine.

Rat. Isolation of optically inactive and laevorotatory forms of 1 2-dihydroxy-1 2-dihydronaphthalene. The rat urine was treated in the same way as the rabbit urine and again two compounds were isolated. The optically inactive product was identical in physical properties with the corresponding rabbit metabolite, but in this case the optically active form was laevorotatory and appeared to be identical with the compound isolated by Young (1947). It was recrystallized from benzene giving colourless plates, m.p. 126°, $[\alpha]_D^{22}$ -158° (c, 0.5% in ethanol) (Found C, 74.2, H, 6.3. Calc. for $C_{10}H_{10}O_2$ C, 74.1, H, 6.2%).

Acetylation. The optically inactive diol from rabbit urine (50 mg) was acetylated in 0.25 ml. acetic anhydride and 0.25 ml. pyridine at room temperature and the diacetate obtained was crystallized from light petroleum (Found C, 68.8, H, 5.8. $C_{14}H_{14}O_4$ requires C, 68.3, H, 5.7%). The other three samples of diol were treated in the same way and acetates with the properties shown in Table 1 were obtained. Each acetate was hydrolyzed, as shown in Table 1.

The diacetate (20 mg) was heated in a boiling water bath for 15 min in 1 ml. 0.7N ethanolic KOH, cooled and a large volume of water added. The precipitate was extracted by

shaking with ether, and the solid obtained on evaporation recrystallized from benzene yielding the parent diol in each case, the melting points are given in Table 1

Table 1 *Properties of acetates of diols from fractions of rabbit and rat urine*

Diol	M.p of acetate (°)	$[\alpha]_D^{22}$ of acetate in ethanol (c, 0.5-1.0%) (°)	M p of diol obtained on hydrolysis of acetate (°)
Rabbit, racemate	73	0	100
Rabbit, optically active form	77	437	126
Rat, racemate	73	0	101
Rat, optically active form	77	-436	126
Rat, optically active form (Young, 1947)	78-79	-423	—

Dehydration with acid to 1-naphthol All four diols behave in a similar manner on treatment with acid, giving theoretical yields of 1 naphthol, identified by mixed melting point, by the colour of the dye formed with diazotized *p* nitraniline and by mixed melting point of the acetate

Rate of dehydration The colour produced by coupling a phenol with a diazotized amine was also utilized to study the rate of dehydration by acid at 100°. Samples, each of

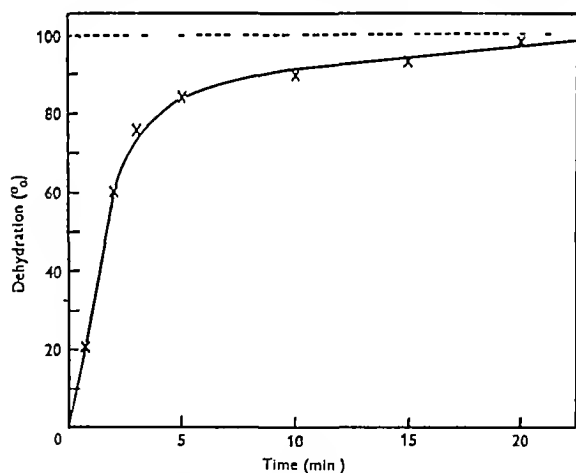


Fig 1 Dehydration of racemic 1,2-dihydroxy 1,2-dihydronaphthalene in *N* HCl at 100°

1 ml., of a solution containing a known amount of optically inactive diol from rabbit urine dissolved in 5% aqueous ethanol and 1 ml *N* HCl were kept in sealed tubes at 100° for various periods of time. The solutions were then cooled and made alkaline by the addition of 5 ml *N* NaOH, 2 ml 0.01M *p* nitrobenzenediazonium chloride solution were added, and the purple colour produced measured on a photoelectric colorimeter, a standard solution of 1-naphthol being used for comparison. The reaction proceeded as shown in Fig 1, giving an almost theoretical yield of 1 naphthol. The reaction was half complete in 1.6 min, whence $k_{100} = 0.43$

Oxidation (1) With potassium permanganate The optically inactive diol from rabbit urine (50 mg) and the dextrorotatory form from rabbit urine gave *o* phthalic acid as described by Young (1947)

Oxidation (2) With lead tetraacetate A solution of 29.4 mg diol in 10 ml glacial acetic acid was added in 1 ml portions to tubes each containing 1 ml of approx 0.1N lead tetraacetate solution in glacial acetic acid, all solutions being kept in a water bath at 20°. The oxidation was stopped at intervals by the addition of 2 ml of iodide solution (2% (w/v) KI, 25% (w/v) Na acetate) and the I_2 liberated titrated against standard 0.01N- $Na_2S_2O_3$, so that the rate of oxidation could be followed. Glycols with adjacent hydroxyl groups in the *cis* position are usually oxidized more rapidly than the *trans* isomers by lead tetraacetate, 1 mol of diol requiring 1 mol of lead tetraacetate (Orregee, Kraft & Rank, 1933). The reaction proceeded rapidly, but no authentic *cis* or *trans* derivative was available for direct comparison. The oxidized solutions gave a positive test for an aldehyde with Schiff's reagent, but the aldehyde was not isolated. All four preparations of the diol reacted at about the same rate with lead tetraacetate. Naphthalene did not react under the same conditions. The time taken for 50% oxidation was 0.8 min, $k_{20} = 127$. This would indicate that the compounds are either all *cis* or all *trans*.

Hydrogenation to a dihydroxytetrahydronaphthalene The compounds were hydrogenated by shaking ethanolic solutions in an atmosphere of H_2 with Pd + $SrCO_3$ catalyst. The hydrogenation products were recrystallized from benzene, and shown to be 1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene (IV). Analysis of product from the inactive diol isolated from rabbit urine found C, 73.2, H, 7.1. $C_{16}H_{12}O_2$ requires C, 73.2, H, 7.3%.

This hydrogenation was carried out for all the specimens of diol and compounds and the properties shown in Table 2 were obtained.

Table 2 *Properties of 1,2-dihydroxy 1,2,3,4-tetrahydronaphthalenes*

	M p (°)	$[\alpha]_D^{22}$ in ethanol (c, 0.5-1.0%) (°)	M p of $KMnO_4$ oxidation product (°)
Rabbit, hydrogenated inactive diol	111	0	160
Rabbit, hydrogenated optically active diol	113	-62	161
Rat, hydrogenated inactive diol	111	0	161
Rat, hydrogenated optically active diol	114	+64	163

The laevorotatory form gave a hydrogenation product identical with that described by Young (1947).

Dehydration of hydrogenated derivative to β tetralone All the four compounds gave a positive test for 2 keto-1,2,3,4-tetrahydronaphthalene (Straus & Rohrbacher, 1921), after boiling with HCl. A few mg of the compound were heated with 1 ml *N*-HCl in a boiling water bath for 15 min and then cooled. On addition of a few drops of ethanol and 2 ml of ether and shaking with excess 10N NaOH a dark blue colour developed in the ether layer.

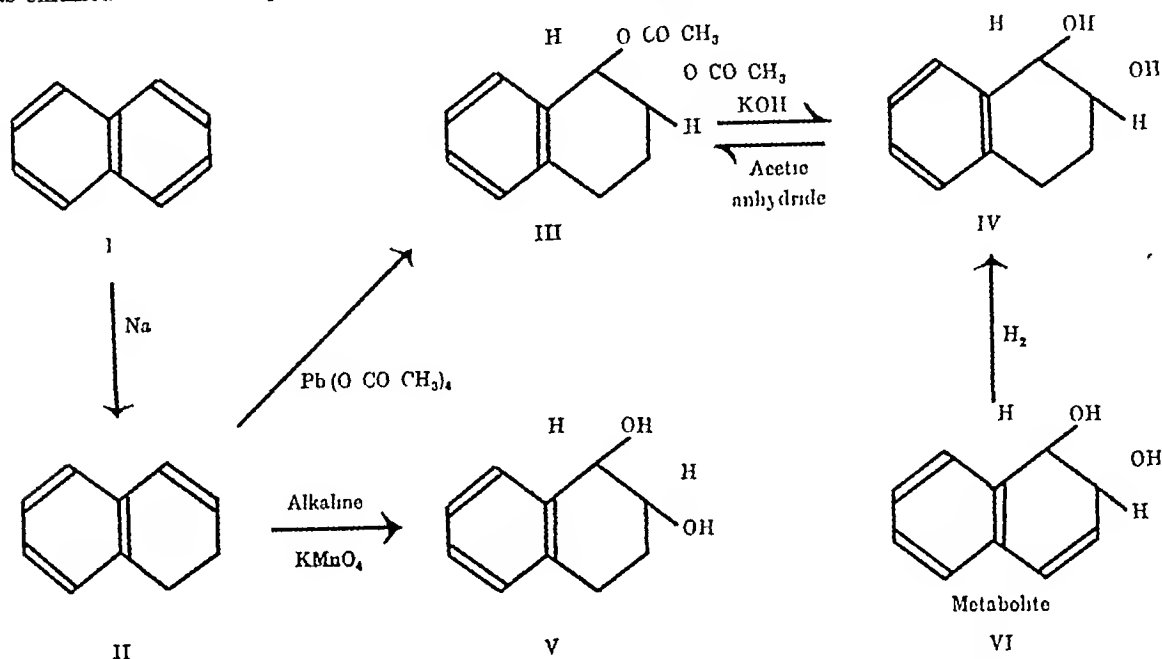
Oxidation of hydrogenated derivative All the forms of the hydrogenation product gave β 2 carboxyphenyl propionic acid on oxidation with KMnO_4 , as found by Young (1947) with the laevorotatory product from rat urine

Spatial configuration The *cis* and *trans* forms of 1 2 dihydroxy-1 2 3 4 tetrahydronaphthalenes were synthesized for comparison with the hydrogenated metabolite

Naphthalene (I) was reduced with Na to give 1 2-dihydronaphthalene (II) (Straus & Lemmel, 1921), and this was oxidized to *cis* 1 2-dihydroxy 1 2 3 4-tetrahydro

correspond to the *trans* isomer in physical properties (see Table 3) The melting point of the mixture of the *trans* isomer and the hydrogenated metabolite was not depressed

(2) Colour reaction with potassium triacetyl osmate (Criegee, Marchand & Wannowius, 1942) When a few drops of a solution of this reagent in acetic acid are added to a substance with the *trans* configuration there is no change from the blue



naphthalene (V) by KMnO_4 (Straus & Rohrbacher, 1921) and to the acetate of the *trans* isomer (III) with lead tetraacetate (Criegee, 1930) The acetate was hydrolyzed to give inactive *trans* 1 2 dihydroxy 1 2 3 4 tetrahydronaphthalene (IV)

Evidence in favour of the hydrogenated metabolite being the *trans* isomers (1) The diacetates of the above isomers and of the hydrogenated optically

colour of the solution, but when added to a *cis* isomer a colour change takes place in a few minutes

When the reagent was added to the synthetic *cis*-dihydroxytetrahydronaphthalene the colour changed from blue to purple and then to grey, but when added to the synthetic *trans* isomer or the hydrogenated metabolite there was no change in colour All preparations of the excreted diols themselves gave no colour change, indicating that they had the *trans* configuration

(3) **Rate of oxidation by lead tetraacetate** These measurements, carried out as described above for the diols, but using specimens of dihydroxytetrahydronaphthalene, are illustrated in Fig 2 The velocity constants, k_{20} , were calculated for the reaction between lead tetraacetate and each of the 1 2-dihydroxy-1 2 3 4-tetrahydronaphthalenes, assuming it to be a bimolecular reaction, and are shown in Table 4

Thus the hydrogenated metabolite is identical with the synthetic *trans*-1 2-dihydroxy-1 2 3 4-tetrahydronaphthalene This shows that the hydroxyl groups in the natural metabolite must have the *trans* configuration, and completely and independently proves the structure of the metabolites to be that of *trans*-1 2-dihydroxy-1 2-dihydronaphthalene (VI) In spite of the rapidity of

Table 3 Melting points of 1 2 dihydroxy-1 2 3 4 tetrahydronaphthalenes

	Synthetic (°)		Hydrogenated metabolite (optically inactive) (°)
	<i>cis</i>	<i>trans</i>	
Melting point	100 (100-101)*	111 (111.5)†	111
Melting point when mixed with hydrogenated metabolite	80-95	111	—
Melting point of diacetate	78 (78-79)*	83 (84)*	83

* Straus and Rohrbacher (1921) † Criegee (1930)

inactive rabbit diol were also prepared for comparison, and the hydrogenated metabolite appears to

reaction of the metabolites with lead tetraacetate, which suggests that the hydroxyl groups might have a *cis* configuration, the *trans* structure seems to be established. Unless known *cis* and *trans* derivatives

Table 4 Values of k_{20} for interaction of lead tetraacetate and 1,2-dihydroxy-1,2,3,4-tetrahydronaphthalenes

Product	Time taken for 50% oxidation (min)	k_{20} (Found)	k_{20} (from Criegee <i>et al</i> 1933)
Synthetic <i>cis</i> compound	15	37.7	40.2
Synthetic <i>trans</i> compound	30	1.89	1.86
Hydrogenated metabolites			
From rabbit, $[\alpha]_D^{20} -62^\circ$	31	1.80	—
From rabbit, $[\alpha]_D^{20} 0^\circ$	37	1.65	—

are available for comparison the rate of reaction with lead tetraacetate cannot be used to indicate the configuration of isomeric glycols of this type. In the case of the 9,10-dihydroxy-9,10-dihydrophenanthrene (Boyland & Wolf, 1948) the evidence suggests that the *trans* derivative is oxidized more quickly than the *cis* derivative, which is contrary to the findings of Criegee *et al* (1933), in the oxidation of a number of other glycols.

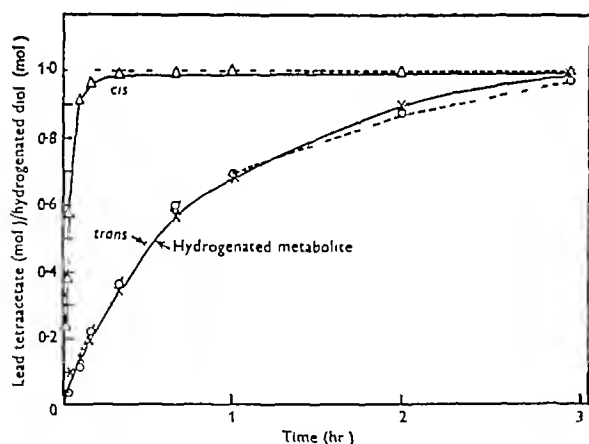


Fig. 2 Oxidation of 1,2-dihydroxy-1,2,3,4-tetrahydronaphthalenes by lead tetraacetate at 20°

The nature of the optically inactive preparations
The 1,2-dihydroxy-1,2-dihydronaphthalene molecule contains two asymmetric carbon atoms, and four stereoisomers are therefore possible, viz *dextro-cis*, *laevo-cis*, *dextro-trans* and *laevo-trans*. None of the products isolated gives the reaction for *cis* glycols, so that the active forms are *dextro-trans* from rabbit urine and *laevo-trans* from rat urine. Although the *cis* forms would have two hydroxyl

groups in the meso positions they would not be optically inactive by internal compensation as the molecule is not symmetrical. The two optically inactive preparations obtained from rat and rabbit urine were identical in all respects, and there was no depression of melting point on mixing the products from the two species. A product identical in physical properties was obtained by mixing the *dextro* and *laevo* forms. Thus 5 mg *laevo*-diol (from rat urine) and 5 mg *dextro* diol (from rabbit urine) each dissolved in 0.5 ml ethanol were mixed and the solvent allowed to evaporate. The residue was recrystallized from benzene and had m.p. 100° (alone and when mixed with a sample of inactive diol isolated from rabbit urine).

Apart from this experimental demonstration that the inactive forms isolated are identical with an artificially prepared racemate they must be so on theoretical grounds. They are *trans* forms of 1,2-dihydroxy-1,2-dihydronaphthalene in which the hydroxyl groups cannot be symmetrically disposed, and, as optically active forms of these are known, the molecule cannot be optically inactive by internal compensation. These inactive preparations must therefore be racemic forms.

The optical activity of different preparations of the diol varied, and the material extracted from rabbit urine usually appeared to contain relatively more of the racemic form. As racemic compounds are not usually produced in biological processes, the racemization probably occurs after formation, either in the process of excretion or isolation. The racemic form is not likely to arise by a Walden inversion on hydrolysis of an ester as that should give a *cis* form unless both hydroxyl groups were esterified.

The racemization process was never observed experimentally. After keeping the *laevo* form dissolved in rat urine, 0.2 M- Na_2HPO_4 or N-NaOH at 20° for 24 hr it could be recovered by ether extraction without change in optical activity. Attempts to resolve the racemate by chromatographic absorption on a column of lactose and development with benzene were unsuccessful.

The metabolic product of anthracene

The 1,2-dihydroxy-1,2-dihydroanthracenes isolated by Boyland & Levi (1935) from the urine of rats and rabbits have been found to behave as *trans* glycols in the reaction with triacetyl osmic acid (Criegee *et al* 1942). In the light of experience with these compounds this reaction would seem to be a better diagnostic test for *cis* and *trans* glycols than the rate of reaction with lead tetraacetate. The anthracene metabolites are, therefore, probably *trans*-1,2-dihydroxy-1,2-dihydroanthracenes, in spite of the fact that they react rapidly with lead tetraacetate (Boyland & Shoppee, 1947).

Examination of the naphthalene metabolite from rabbit urine indicated that a mixture of a racemate and an optically active form occurred with considerable variation in the proportions of the two forms present. In view of this result and the fact that the rabbit metabolite, 1,2-dihydroxy-1,2-dihydroanthracene, had been first described with $[\alpha]_D +16.2^\circ$ (Boyland & Levi, 1935) and later with $[\alpha]_D 0^\circ$ (Boyland & Shoppee, 1947), the metabolites of anthracene were reinvestigated.

The optically inactive material was obtained by sublimation at reduced pressure and this process appears to yield the racemate. By crystallization or distribution between solvents the optically active forms can also be obtained. By repeated crystallization of the diacetates of mixed 1,2-dihydroxy-1,2-dihydroanthracenes obtained from rabbit urine, twice from an ether light petroleum (b.p. 40–50°) mixture and then from ethanol, optically active material with $[\alpha]_D^{20} +345^\circ$ (c, 0.7% in dioxan) and m.p. 150° was obtained. This did not change in physical properties on recrystallization from ethanol. This acetate was hydrolyzed with ethanolic KOH to yield *dextro* 1,2-dihydroxy-1,2-dihydroanthracene, m.p. 162°, $[\alpha]_D^{22} +154^\circ$ (c, 0.5% in dioxan). From the mother liquors of the crystallization optically inactive 1,2-diacetoxy-1,2-dihydroanthracene with m.p. 121° was obtained. This, on hydrolysis, gave an optically inactive 1,2-dihydroxy-1,2-dihydroanthracene, m.p. 184°. In the light of these findings the products first described (Boyland & Levi, 1935) would appear to have been mixtures of racemic and optically active forms while the preparation investigated by Boyland & Shoppee (1947) was a racemate.

The 1,2-dihydroxy-1,2-dihydroanthracene isolated from rat urine was subjected to the same procedure as was carried out with the rabbit product. The optically active 1,2-diacetoxy-1,2-dihydroanthracene crystallized from aqueous ethanol in rectangular plates, m.p. 149°, $[\alpha]_D^{22} -342^\circ$ (c, 0.7% in dioxan). This was hydrolyzed with hot ethanolic KOH to give 1,2-dihydroxy-1,2-dihydroanthracene which crystallized from benzene in flat needles, m.p. 162°, $[\alpha]_D^{22} -149^\circ$ (c, 0.5% in dioxan). From the mother liquors from the crystallization of the diacetate there was obtained material which crystallized from aqueous ethanol in rosettes, m.p. 122°, $[\alpha]_D^{22} -6^\circ$ (c, 0.5% in dioxan). This was hydrolyzed to yield an optically inactive 1,2-dihydroxy-1,2-dihydroanthracene which crystallized from benzene in

irregular plates, m.p. 184°, $[\alpha]_D^{22} 0^\circ$ (c, 0.2% in dioxan). The laevorotatory and dextrorotatory forms of 1,2-dihydroxy-1,2-dihydroanthracene obtained by crystallization appear to be enantiomorphous. A mixture of equal quantities (5 mg) of the two pure optically active forms gave a solution which was optically inactive and the solution on evaporation yielded crystalline material, m.p. 182°, which appeared to be identical with the optically inactive products isolated by fractional crystallization of the metabolites.

These results show that the 1,2-dihydroxy-1,2-dihydroanthracenes previously described were not optically pure, but all the unfractionated preparations of the diols of anthracene or naphthalene obtained as metabolites from rat urine have been laevorotatory while those obtained from rabbit urine have been either dextrorotatory or optically inactive. The difference in metabolism may be associated with the fact that the injection of a carcinogenic hydrocarbon such as 1,2,5,6-dibenzanthracene yields tumours in rats but not in rabbits.

The fact that *trans* isomers are produced in the biological oxidation means that the products are like those produced by oxidation with lead tetraacetate or iodosobenzene, and not like those formed by oxidation with osmic acid or peracetic acid.

SUMMARY

1. A 1,2-dihydroxy-1,2-dihydronaphthalene (in dextrorotatory and inactive forms) was isolated from the urine of rabbits, and the same compound (in laevorotatory and inactive forms) from the urine of rats, after the animals had been dosed with naphthalene. The hydroxyl groups of these diols have been proved to have the *trans* configuration.

2. The 1,2-dihydroxy-1,2-dihydroanthracenes isolated as metabolites of anthracene have been isolated in optically pure forms and these are also probably *trans* compounds.

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The Excretion of Synthetic Oestrogens as Ethereal Sulphates and Monoglucuronides in the Rabbit and in Man

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Mazur & Schorr (1942) found that when stilboestrol (4,4'-dihydroxy- $\alpha\beta$ -diethylstilbene) was administered to the rabbit the main product excreted in the urine was stilboestrol monoglucuronide, in amounts corresponding to about 30% of the dose. This finding was confirmed by Wilder Smith & Williams (1948) and extended to hexoestrol and dienoestrol. Prof R T Williams (private communication) found that, in the rabbit, probably up to about 10% of administered synthetic oestrogen was excreted as the ethereal sulphate. This figure was based on increases of total urinary ethereal sulphate on administering synthetic oestrogen, increases which were, however, not always unequivocal.

In man, the excretion of stilboestrol as the monoglucuronide in substantial amounts was reported by Dodgson & Williams (1948) and by Wilder Smith (1947), whilst detection of a small excretion as the ethereal sulphate was claimed by Wilder Smith (1947).

Further experiments on excretion of synthetic oestrogens by the rabbit and by man are reported here together with the methods used for separation and assay of the ethereal sulphate, monoglucuronide and free oestrogen in urines.

METHODS

In the case of man the oestrogen was given in the form of tablets by mouth and the urine collected for 48 hr and preserved with toluene. In the rabbit the oestrogen was given in solution in ethyl lactate by stomach tube, the urine was collected for 4 days and preserved with toluene. In both cases the urines were stored at 3° until use. The period of storage never exceeded 1 week.

The method we have used for the ethereal sulphate and other determinations has the disadvantage that it is dependent on bioassay. It has been found that if slightly acidified urine or water containing hexoestrol, hexoestrol disulphate and hexoestrol monoglucuronide is extracted with ether the hexoestrol and hexoestrol monoglucuronide pass almost quantitatively into the ether layer. They may be separated from each other by extraction of the ether solution with aqueous NaHCO_3 , in which the glucuronide is soluble, followed by dilute aqueous NaOH in which the hexoestrol is soluble. The urine or water remaining contains the ethereal sulphate from which the free oestrogen may be liberated by hydrolysis by acid, the amount of the ethereal sulphate originally present was determined by bioassay.

In order to carry out the preliminary recovery experiments it was necessary to prepare the pure monoglucuronide and disulphate of hexoestrol. The monoglucuronide was obtained from the metabolism experiments reported previously (Simpson & Wilder Smith, 1948) while the disulphate (3,4-diphenylhexane 4',4''-disulphuric acid) was prepared by treating the free phenol at -10° in carbon disulphide and dimethylaniline with chlorosulphonic acid (cf Burkhardt & Lapworth, 1926, Short & O'Leary, 1946) followed by crystallization from $2N$ NaOH .

Preliminary recovery experiments

(a) *Preparation of solutions* The synthetic mixtures for test were made up by weighing out the hexoestrol monoglucuronide and the sodium salt of hexoestrol disulphate and adding them to a known volume of water or urine. The free oestrogen was weighed out and dissolved in a known volume of ethanol, the required portion was then pipetted from this solution directly into the centre of the prepared aqueous solution of sulphate and monoglucuronide. Care was taken to prevent precipitation losses due to the insolubility of the free oestrogen.

Hexoestrol and its derivatives were used in preference to those of stilboestrol or dienoestrol on account of their greater stability (Wilder Smith & Williams, 1947).

(b) *'Working up' procedure* The sample was acidified with HCl until just acid to Congo red paper and was then well shaken three times with equal volumes of ether. Foams were broken by adding traces of surface active agents (e.g. Cal solene). In general, the use of fairly large quantities of ether tended to reduce emulsion formation. Residual foams after this treatment were broken by filtration. Salt may not be used to break foams since the aqueous layer left after ether extraction is used directly for bioassay after suitable dilution.

After the three hand extractions the aqueous layer was extracted for 24 hr in a continuous ether extractor and the combined extracts evaporated to dryness, finally under reduced pressure. The residue was treated with 40 ml saturated aqueous NaHCO_3 solution and the resultant mixture extracted with 3×40 ml ether. The bicarbonate layer, containing the monoglucuronide, was made acid to Congo red paper with HCl , extracted with 5×40 ml ether, the ether removed by evaporation and the residue made up to 100 ml with acetone for bioassay.

The ethereal solution which had been extracted with bicarbonate solution was then washed three times with $2N$ - NaOH and the aqueous layer, containing the free oestrogen, acidified to Congo red paper, extracted with ether three times and the ether extract made up in the same way for bioassay as the ether extract from the acidified bicarbonate layer.

The remaining original aqueous layer (containing the ethereal sulphate) from the continuous ether extraction, was refluxed for 1 hr after the addition of 1% by volume of concentrated HCl, neutralized with dilute NaOH and assayed on rats directly after suitable dilution with water. Where the concentration of oestrogen present was too low for direct assay after this procedure, the solution was extracted with ether and the concentrated extract assayed.

(c) *Bioassay* The extracts of free oestrogen and monoglucuronide in standard volumes in ethanol or acetone were diluted with water to give doses corresponding to recoveries of 10, 25, 50, 75 and 100%. Thus a rough range of recovery efficiency was obtained which was later narrowed by intermediate dosing.

In the case of ethereal sulphate excretion in actual metabolism experiments much lower dose levels were required than in the case of the recovery experiments. In these cases the two nearest dose levels giving 80–100% and 0% response on five rats were reported in the Tables since the total excretion was so low that it was obviously not worth while narrowing the range to give figures which would allow of statistical treatment. The procedure of subcutaneous injection and vaginal smearing was the standard one used in this Institute (Wilder Smith & Williams, 1948). It will be seen that by this method only very approximate figures can be given.

Table 1 *Recovery of hexoestrol, hexoestrol monoglucuronide and sodium hexoestrol disulphate from water and urine**

Medium used	Recovery of hexoestrol (1 mg) (%)	Recovery of Na hex-oestrol disulphate (10 mg) (%)	Recovery of hexoestrol monoglucuronide (10 mg) (%)
Water (1 l)	67 (49–91)	48 (33–71)†	80 (63–105)
Normal male urine (1 l)	91 (77–117)	65 (51–84)	104 (89–124)
Normal male urine (1 l)	88 (73–106)	74 (64–85)	—
Normal male urine (1 l)	103 (79–135)	82 (57–114)	108 (90–129)

* The mean responses and fiducial limits (in parentheses) were determined by the methods given in Fisher & Yates (1943).

† The low figure was due to insufficient hydrolysis.

RESULTS

Table 1 shows the results of some recovery experiments of hexoestrol monoglucuronide, hexoestrol disulphate and hexoestrol added to water and to urine. Tables 2 and 3 give the results of metabolism

Table 2 *Ethereal sulphate excretion of synthetic oestrogens in the rabbit*

Rabbit no	Dose (mg)	Excretion as ethereal sulphate* (%)	Monoglucuro nide excretion for comparison† (%)
Stilboestrol			
R 30	500	0.04-0.2	} (30-40)
R 30	500	0.04-0.2	
R 30	240	0.04-0.4	
R 30	240	0.08-0.4	
R 20	275	0.1-0.4	
R 20	275	0.04-0.1	
Hexoestrol			
R 8	500	0.1-0.5	} (30-46)
R 9	500	0.05-0.1	
Dienoestrol‡			
R 6	500	0.1-0.5	} (24-48)
R 7	500	0.1-0.5	
R 8	400	0.2-0.5	
R 9	525	0.2-0.5	

* The lower percentage quoted gave 80–100% response in five rats, while the higher figure gave 0% response in five rats, these figures are not fiducial limits.

† Cf. Wilder Smith & Williams (1948).

‡ The bioassay calculation for ethereal sulphate was made on the basis that the dienoestrol was cyclized to the corresponding indene during acid hydrolysis (cf. Hausmann & Wilder Smith, 1948).

experiments carried out in man and the rabbit. Excretion of synthetic oestrogens as glucuronides in the rabbit has been reported previously (Mazur & Schorr, 1942; Wilder Smith & Williams, 1948).

Table 3 *Ethereal sulphate and monoglucuronide excretion of synthetic oestrogens in man*

Case	Patient no	Oestrogen used	Dose (mg)	Percentage* excreted		
				As ethereal sulphate	As mono glucuronide	As free oestrogen
Prostatic cancer	1	Stilboestrol	25	1–5	50–60	1–2
"	1	"	20	3–9	10–25	1–5
Normal woman	4	Hexoestrol	5	1–5	75–100	10
"	4	"	15	<0.5	25–50	<1
"	5	"	5	<1.5	25–50	<1
"	5	"	50	<0.2	10–20	<1
"	6	"	10	0.1–1	30–40	0.1–1
"	7	"	10	0.1–1	25–50	0.1–1

* The lower percentage quoted gave 80–100% response in five rats, while the higher figure gave 0% response in five rats, these figures are not fiducial limits.

DISCUSSION

In preliminary experiments it was found that, if the continuous ether extraction of the urine or water was continued for much more than 24 hr, low recoveries of sulphate with correspondingly higher recoveries of free oestrogen were obtained. This is due to the fact that under the acid conditions of the experiment, the ethereal sulphate is hydrolyzed to the free oestrogen which is then extracted by the ether. The somewhat high apparent recoveries of glucuronide are due to the slight solubility of free oestrogen in saturated aqueous bicarbonate. The high biological activity of the free oestrogen compared with that of the glucuronide makes noticeable the presence of even a trace of free oestrogen. During preliminary experiments also it was found that low sulphate figures resulted unless care was taken to ensure complete hydrolysis of the ethereal sulphate before bioassay.

From Table 2 it is seen that the ethereal sulphate excretion in the rabbit was very low. Although the method was worked out for hexoestrol and its derivatives the similarity in chemical and physical properties of stilboestrol and its derivatives make it likely that the method can be applied in their case also without much probability of serious error. In the case of dienoestrol it is necessary to make an additional correction to compensate for the conversion of dienoestrol to the corresponding indene (Hausmann & Wilder Smith, 1948) under the acid conditions used for the hydrolysis of the ethereal sulphate.

It can be seen from Table 3 that large variations in glucuronide and ethereal sulphate excretion occur in man as in the case of the rabbit. Owing to the small dosage of oestrogen given in the case of man the experimental errors are likely to be larger. Nevertheless, it is quite clear that the excretion as monoglucuronide in man is much larger than the excretion as free oestrogen or ethereal sulphate.

In both the human subject and in the rabbit fairly high recoveries of monoglucuronide are obtained, both by our method and that of Dodgson & Williams (1948), but we have consistently found that, if the urine of patients treated with stilboestrol or hexoestrol is hydrolyzed under conditions known to give high yields of the free oestrogen

from the glucuronide, only about 10–15% of the original oestrogen administered can be detected afterwards as the free oestrogen. Thus, urines showing the presence of about 40–50% of the original oestrogen as monoglucuronide by the method given above will, on hydrolysis and redetermination as free oestrogen in the crude urine, show only about 10–15% present. Although it is known that the monoglucuronide figure determined by our method tends to be high, yet this is scarcely sufficient to account for the discrepancy, for which we can offer at present no other explanation than to suggest that some other oestrogen, soluble in sodium bicarbonate, may be present which has a higher oestrogenic potency than that of the monoglucuronide.

The figures reported show that the general pattern of excretion of synthetic oestrogens is similar both for the rabbit and man in that the vastly increased doses used in animals for these metabolism experiments do not give a substantially changed picture from that obtained in therapeutic dosing in man.

SUMMARY

1 A method of separating the ethereal sulphates of certain synthetic oestrogens from their monoglucuronides and free forms is described.

2 A small excretion of synthetic oestrogen as ethereal sulphate has been found in both man and the rabbit.

3 The method used for working up gives reasonably quantitative results on known synthetic mixtures in urines, so that there is little doubt that the comparatively low overall recoveries obtained in metabolism experiments are not due entirely to inadequate technique, but do indicate a real metabolic breakdown.

4 Results obtained by determination of the metabolized oestrogen as the monoglucuronide are consistently higher than those obtained by hydrolysis of all conjugated forms of oestrogen present followed by determination as free oestrogen. A possible explanation for this is offered.

We are indebted to the Council of the Middlesex Hospital for the provision of laboratory facilities for this work, to Mrs D Culhford and Mr B D Shepherd for technical assistance, to Dr W Hausmann for the preparation of the synthetic oestrogen disulphates and to the Medical Research Council for a grant.

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The Formation of Hydrogen Carriers by Haematin-catalyzed Peroxidations

2 SOME REACTIONS OF ADRENALINE AND ADRENOCHROME

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Green & Richter (1937) found that adrenaline induces a vigorous oxygen uptake when added in low concentrations to the reconstructed lactic and malic dehydrogenase systems of heart muscle. This effect was found to be due to the oxidative formation from adrenaline of a substance which they identified and named adrenochrome. The formation of adrenochrome depended on the presence in their enzyme preparations of a factor which they considered very probably to be cytochrome *c*. It was further found that in the presence of cytochrome *c* the effect was stimulated by hydrogen peroxide. They assumed that the role of hydrogen peroxide was the oxidation of ferrocytochrome *c*, and that ferricytochrome *c* oxidized adrenaline to adrenochrome. Cytochrome *c* and hydrogen peroxide thus exerted what Green & Richter called a 'trigger' effect, evidently by hastening the formation of adrenochrome. The addition of low concentrations of adrenochrome itself to the enzyme-adrenaline system also caused a 'trigger' effect, the reaction lag being shortened, but the maximum rate eventually reached not being much affected.

It was established that, once formed, the adrenochrome acted as a carrier in these systems. Green & Richter were not certain whether the reoxidation of the reduced (leuco) adrenochrome was enzymic or autoxidative.

Hermann, Boss & Friedenwald (1946) have shown that 'oxidized adrenaline' solutions are able to take part in a coupled oxidation of ascorbic acid, and that the rate in this case is further stimulated by the addition of cytochrome *c*.

In an earlier paper (Albert & Falk, 1949) the peroxidative oxidation of certain acridine and quinoline compounds, catalyzed by cytochrome *c* or methaemoglobin, was discussed. In the present paper a similar peroxidative oxidation of adrenaline is described, adrenochrome is shown to act as an autoxidizable hydrogen carrier in the oxidation of ascorbic acid, and to catalyze the oxidation of adrenaline itself. These reactions offer a possible explanation of the 'trigger effects' of cytochrome *c* and adrenochrome described by Green & Richter

(1937). They are a further example of the non-specific haematin peroxidative effect, which may also be of importance *in vivo*.

EXPERIMENTAL

Materials

Water for all purposes was double distilled from an all glass still. Adrenaline was pure (—) adrenaline, m.p. 213° (decomp.), kindly presented by Burroughs, Wellcome and Co., Sydney. It was stored under N₂, and weighed amounts were dissolved immediately before use in 1 equiv. of 0.1N-HCl and made up to the required volume.

Adrenochrome was prepared from adrenaline by the method of MacCarthy (1945, 1946), quoted by Randall (1947), melting point, after crystallizing from dry methanol-formic acid, was 130° (decomp.) (Found C, 60.1, H, 5.1, N, 7.6. Calc. for C₉H₉O₃N: C, 60.3, H, 5.1, N, 7.8%). The monoxime of this adrenochrome was prepared and, as reported by Veer (1942*a*), it was found that its melting point rose as the substance lost water. After drying quickly on porous tile it melted sharply, with decomposition, at 175° and at 180° (decomp.) after drying *in vacuo* over CaCl₂. Solutions of adrenochrome in water were prepared immediately before use.

The ascorbic acid was pure, solutions were made in distilled water, with the addition of 1 equiv. of 0.1N-NaOH, immediately before use. H₂O₂ solutions were prepared by diluting 100 vol. peroxide. Schering Kahlbaum copper-free cysteine was used. The melting point of the quinol was 286°, and that of the catechol, 104°. The cytochrome *c*, methaemoglobin and catalase were prepared as described previously (Albert & Falk, 1949).

Methods

O₂ uptakes were measured in Warburg respirometers at pH 7.0 and 25 or 37.5°. The gas phase was usually air, and the shaking rate 120 per min. The total volume of liquid per flask was 2.0 ml, and the final molarity of the phosphate buffer was 0.12.

RESULTS

The effect of haematin compounds on the oxidation of adrenaline

Fig. 1 shows the effect of cytochrome *c* on the rate of oxidation of adrenaline solutions at pH 7 and 37.5°. The rate gradually increased in all cases to

a maximum, which was maintained steadily for some time, the lag, however, was decreased in proportion to the amount of cytochrome *c* added. In these experiments the red colour, characteristic of adrenochrome, appeared at about the end of the lag

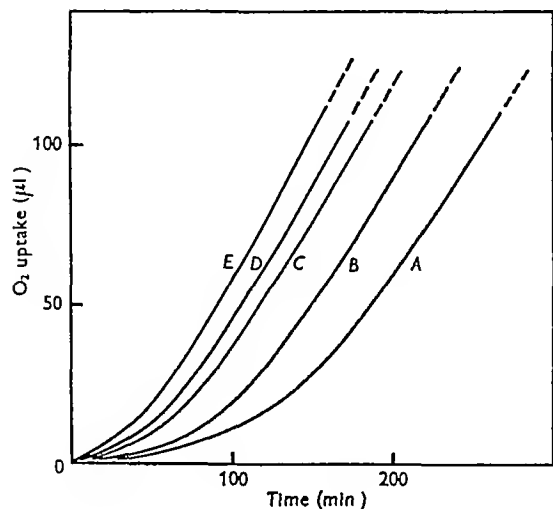


Fig 1 The effect of cytochrome *c* on the oxidation of adrenaline. O_2 uptake was measured at pH 7 and 37.5° . Each flask contained 2.0 mg of adrenaline. Cytochrome *c* in curves A-E, 0, 0.1, 0.2, 0.3 and 0.5 mg respectively.

After 10 hr oxygen uptake was still occurring in all the flasks at rates only slightly lower than the maximum. At this time, the depth of the colour was greater the more cytochrome *c* had been added. Finally the colour became very dark, suggesting melanin formation. There was no output of carbon dioxide. The adrenaline was eventually oxidized past adrenochrome, even in the absence of cytochrome *c* (theoretical oxygen uptake for 1 mg adrenaline to adrenochrome, 122 μ l).

Table 1 Peroxidative catalysis of the oxidation of adrenaline

(O_2 uptakes were measured at pH 7 and 37.5° . Each flask contained 2.0 mg adrenaline ($5.6 \times 10^{-3}M$). Sufficient catalase was added to decompose completely 2.0 ml of $m-H_2O_2$ in 10 min, the cytochrome *c* was $0.9 \times 10^{-5}M$, the methaemoglobin $0.22 \times 10^{-5}M$, and the H_2O_2 $10^{-3}M$. The lag (time to reach maximum rate) was reproducible to ± 3 min.)

Reagents added	Lag (min)
—	180
H_2O_2	180
Cytochrome <i>c</i>	65
Cytochrome <i>c</i> + H_2O_2	30
Cytochrome <i>c</i> + catalase	140
Methaemoglobin	75
Methaemoglobin + H_2O_2	35
Methaemoglobin + catalase	170

Methaemoglobin reacted similarly to cytochrome *c* (Table 1). In both cases the further stimulation

(shortening of lag) by hydrogen peroxide and the inhibition by catalase suggested a peroxidative mechanism.

Green & Richter (1937) found a very rapid uptake of oxygen in the system adrenaline cytochrome *c*-cytochrome oxidase. Here the adrenaline was simply the substrate for the cytochrome system, when the oxidase was boiled there was no uptake in the first 5 min. The peroxidative effect described above is only revealed when measurements are made for longer periods.

The effect of adrenochrome on the oxidation of adrenaline

Green & Richter (1937) studied the system malate, malic dehydrogenase, coenzyme, cyanide and adrenaline. In the full system small amounts of adrenochrome greatly stimulated the rate of oxygen uptake and the oxidation of adrenaline to adrenochrome. In the absence of malate they found that the addition of adrenochrome did not stimulate the oxygen uptake (during the first 3 min) and did not bring about the oxidation of adrenaline to adrenochrome.

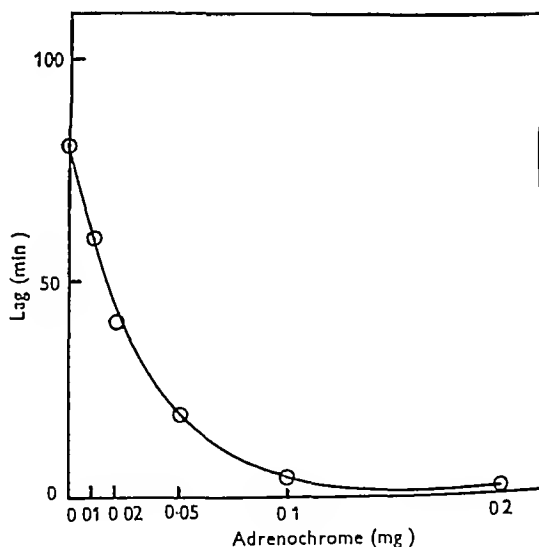


Fig 2 The effect of adrenochrome on the oxidation of adrenaline. O_2 uptake was measured at pH 7 and 37.5° . Each flask contained in the main bulb 2.0 mg of adrenaline, and the adrenochrome solutions were tipped from a side arm. The adrenaline was $5.6 \times 10^{-3}M$ (0.2 mg adrenochrome gave a molarity of 5.4×10^{-4}).

It is now found that adrenochrome shortens the lag in the oxidation of adrenaline in a simple system containing only adrenaline and adrenochrome at pH 7 and 37.5° . The results are shown in Fig 2, the lag was progressively shortened with increasing amounts of adrenochrome. In controls with adrenochrome alone, the oxygen uptake was negligible. The original oxygen-uptake curves were

of the same type as those shown in Fig 1, and only with very high adrenochrome concentrations could any stimulation be observed in the first 3 min

Adrenochrome did not increase the rate of autooxidation of cysteine, catechol or quinol at pH 7 and 25° or 37 5°

The effect of adrenochrome on the oxidation of ascorbic acid

Adrenochrome catalyzes the oxidation of ascorbic acid (Fig 3) In these experiments there was no lag, and the oxygen uptake was linear within certain limits of time

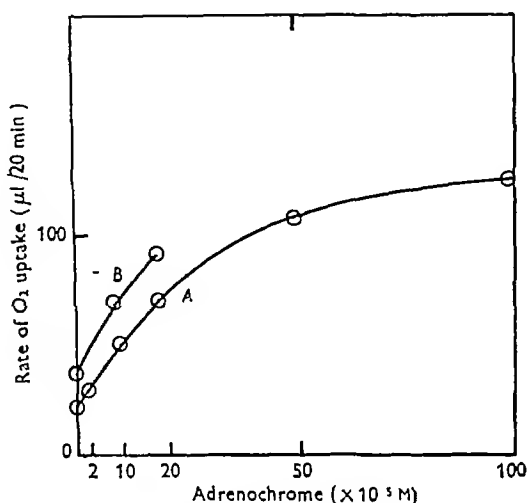


Fig 3 The effect of adrenochrome on the oxidation of ascorbic acid. O₂ uptake was measured at pH 7. Each flask contained in the main bulb 10 mg ascorbic acid (2.8×10^{-3} M). The adrenochrome was tipped from a side arm. Curve A, 25°, curve B, 37 5°

Hermann *et al* (1946) found that 'oxidized adrenaline' solutions had a similar effect on the oxidation of ascorbic acid, and that a further stimulation was caused by cytochrome *c*. The present author has repeated this experiment under the conditions described by Hermann *et al* (1946) with quantitative agreement with their results. These workers considered that the 'oxidized adrenaline' solutions contained 'some unoxidized adrenaline and several oxidation products at different steps towards the maximum possible oxidation'. In this connexion it is noteworthy that with an amount of this solution (prepared according to their method) equivalent to an original 0.4 mg of adrenaline, 3.0 mg of ascorbic acid absorbed only 72 μl of oxygen in 20 min at pH 7.0 and 37 5°. Under similar conditions, but with 0.4 mg of adrenochrome instead of oxidized adrenaline, 280 μl of oxygen were absorbed.

Under no conditions, however, was cytochrome *c* found to stimulate the rate of oxidation of ascorbic

acid in the presence of pure adrenochrome alone. As has been shown above, cytochrome *c* considerably stimulates the oxidation of adrenaline, and it seemed possible that the cytochrome *c* stimulation of Hermann *et al* (1946) was due to the effect of the cytochrome *c* in converting unchanged adrenaline in the solution of 'oxidized adrenaline' to adrenochrome. Provided that the rate was limited by the adrenochrome concentration (cf Fig 3) cytochrome *c* should thus cause a stimulation. Under certain conditions (Table 2) it was in fact found that cytochrome *c* caused a stimulation in the rate of oxidation of ascorbic acid in the presence of mixtures of adrenochrome and adrenaline.

Table 2 Oxidation of ascorbic acid with a mixture of adrenaline and adrenochrome

(O₂ uptakes were measured at pH 7 and 37 5°. Each flask contained 20 mg of ascorbic acid in a side arm, the remainder of the reagents being in the main bulb. Adrenochrome was limiting in Exps 1 and 2. All values are corrected for ascorbate autooxidation (15 μl/10 min). In Exps 1 and 2, the flasks were shaken in the bath for 30 min before tipping in the ascorbic acid.)

	Exp 1	Exp 2	Exp 3	Exp 4
Adrenaline (mg)	0.3	0.3	0	0
Adrenochrome (mg)	0.036	0.036	0	0
Cytochrome <i>c</i> (mg)	0	0.5	0	0.5
Oxidized adrenaline (mg)	0	0	0.4	0.4
O ₂ uptake (μl/10 min)	55, 57	75, 77	20, 20	129, 132

When the ascorbic acid was added, without previous incubation, only a very slight cytochrome *c* stimulation was found under the conditions of Exps 1 and 2. There seems little doubt (Fig 2) that the leucoadrenochrome-adrenochrome system acts as a hydrogen carrier in the oxidation of adrenaline, and it is likely that hydrogen peroxide is formed in the reaction (see p 372). Thus 'oxidized adrenaline' solutions may differ from mixtures of adrenaline and adrenochrome in containing small amounts of hydrogen peroxide. When Exps 1 and 2 of Table 2 were repeated in the presence of 10^{-3} M-hydrogen peroxide, a similar cytochrome *c* stimulation was found without previous incubation.

DISCUSSION

The oxidation of adrenaline

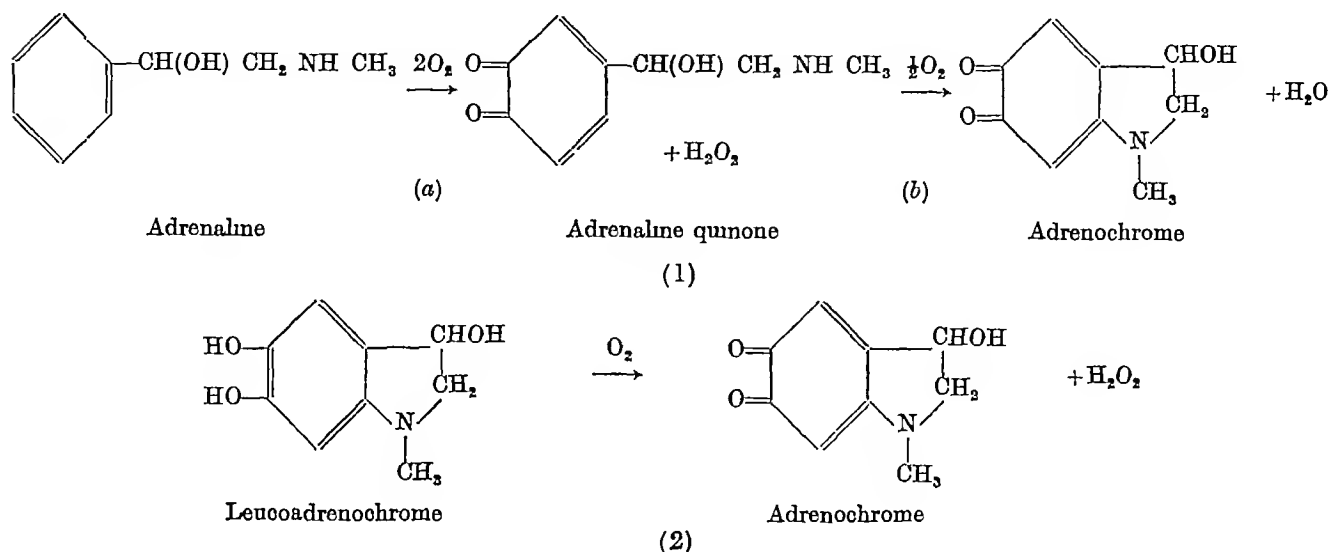
On shaking solutions of adrenaline in air at pH 7.0, a relatively slow, autocatalytic oxidation occurs, presumably involving reactions (1a) and (1b) (see p 372).

Melanin is eventually formed by unknown reactions, probably involving further oxidation and polymerization. The rate, followed by measurement

of oxygen uptake, is typical of an autocatalytic process (Fig 1, curve *A*), it is possible that this is due to adrenochrome, as formed, acting as a hydrogen carrier, being reduced by adrenaline to leucoadrenochrome with reoxidation by oxygen (reaction (2))

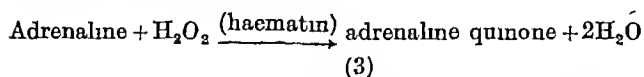
invalid, since it requires the reduction of the adrenochrome by the more electropositive adrenaline (Table 3)

However, Ball & Chen (1933) measured a thermodynamically reversible step in the oxidation of adrenaline in acid solution, but found that at pH 7



This mechanism is supported by the results shown in Fig 2. Reaction (1a) is probably slow compared with (1b), and it is probably (1a) which is catalyzed by the adrenochrome (see below, and cf also LuValle & Weissberger, 1947)

The stimulation by cytochrome c and methaemoglobin of the oxidation of adrenaline is clearly a peroxidative effect, since it is stimulated by hydrogen peroxide and inhibited by catalase. It is probably again reaction (1a) which is catalyzed, becoming now reaction (3)



Autocatalysis here is probably due to two factors, adrenochrome as it is formed catalyzing the oxidation as a hydrogen carrier, and the hydrogen peroxide formed (reaction (2)) contributing to the peroxidative catalysis.

The postulation of adrenochrome as a hydrogen carrier in the oxidation of adrenaline may seem

the oxidant of the system was extremely unstable. There is little doubt that they were dealing with the system $\text{adrenaline} \rightleftharpoons \text{adrenaline quinone}$ (1a), the latter undergoing at pH 7 a very rapid irreversible change with ring closure to yield adrenochrome by oxidation (either directly or after rearrangement to leucoadrenochrome), or by dismutation.

Such a system with an unstable component may be completely oxidized by another much more electronegative system. Thus Ball & Chen (1933) found that at pH 7 one equivalent of adrenaline (E'_0 , pH 7 = +0.388 V) was capable of reducing four equivalents of 2,6-dichlorophenolindophenol (E'_0 , pH 7 = +0.217 V).

The oxidation of ascorbic acid by adrenochrome

In catalyzing the oxidation of ascorbic acid (Fig 3) adrenochrome again acts as a hydrogen carrier. A similar oxidation of the still more electro-

Table 3 *Oxidation-reduction potentials*

Substance	E'_0 at pH 7.0 (V)	Temp (°C)	Reference	Method
Adrenaline	+0.388	30	Ball & Chen (1933)	Oxidative
Catechol	+0.360	30	Ball & Chen (1933)	Oxidative
Quinol	+0.271	30	Ball & Chen (1933)	Oxidative
Leucoadrenochrome	+0.044	20	Wiesner (1942)	Polarographic
Ascorbic acid	-0.066	35.5	Borsook & Keighley (1933)	Potentiometric (method of mixtures) extrapolated to pH 7.0
Cysteine	-0.390	25	Borsook, Ellis & Huffman (1937)	Thermal

negative cysteine might be expected. The oxidation of cysteine is very complex, however (cf Remick, 1943, Borsook *et al* 1937), and E'_0 values at pH 7 as high as +0.06 V have been recorded (Williams & Drissen, 1930).

It is possible that adrenaline may be oxidized in living tissues by non-enzymic mechanisms such as those described above. The importance of adrenochrome has been stressed by Veer (1942*a*), who brought forward evidence that it is biologically active, and that it is a melanin precursor *in vivo*. The closely related pigment hallachrome was found by Friedheim (1932, 1933) to increase the oxygen consumption of erythrocytes, and probably plays a role in the maturation of reticulocytes (Gad, Jacobsen & Plum, 1944). Veer (1942*b*) obtained evidence for somewhat similar action of adrenochrome, in that it augments the leucocyte migration of animal bone-marrow transplantations *in vitro*.

The catalysis by adrenochrome of adrenaline oxidation is analogous to the non enzymic step in the oxidation of tyrosine discussed by Kertész (1948), in that in each case the catalyst is an *o*-quinone. Leucoadrenochrome, however, is very rapidly autoxidized at pH 7, while dihydroxy-

phenylalanine oxidation is rapid only when catalyzed enzymically.

SUMMARY

1 The oxidation of adrenaline by atmospheric oxygen at pH 7 is catalyzed peroxidatively by cytochrome c and by methaemoglobin. Traces of hydrogen peroxide originally formed by autoxidation of adrenaline are later augmented by autoxidation of leucoadrenochrome.

2 The oxidation of adrenaline is catalyzed by adrenochrome, which acts as a hydrogen carrier.

3 Adrenochrome acts as a hydrogen carrier in catalyzing the oxidation of ascorbic acid by atmospheric oxygen. It does not affect the rate of oxidation of cysteine, catechol or quinol at pH 7.

4 The catalysis of the oxidation of ascorbic acid by 'oxidized adrenaline' solutions appears to be due to the adrenochrome content of these solutions.

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The Substrate Specificity of the Tyrosine Decarboxylase of *Streptococcus faecalis*

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Streptococcus faecalis has been shown to possess an enzyme which decarboxylates L-tyrosine and L-3,4-dihydroxyphenylalanine, with the formation of carbon dioxide and the corresponding amines; this enzyme is known as tyrosine decarboxylase (Gale, 1940, Epps, 1944). L-3,4-Dihydroxyphenylalanine is also a substrate of the DOPA decarboxylase of mammalian tissues. This enzyme attacks neither L-tyrosine nor L-phenylalanine (Blaschko, 1939),

but it does decarboxylate L-2,5-dihydroxyphenylalanine, L-*m*-hydroxyphenylalanine and L-*o*-hydroxyphenylalanine (Blaschko & Sloane Stanley, 1948, Blaschko, 1949). The experiments described in this paper were done in order to find out whether 2,5-dihydroxyphenylalanine, *m*-hydroxyphenylalanine and *o*-hydroxyphenylalanine are substrates of the streptococcal tyrosine decarboxylase.

METHODS

Preparation of the enzyme A strain of *Strep faecalis* R (American Type Culture Collection no 8043), given by Prof I C Gunsalus, was used. The organism was maintained as a stab culture in glucose marmite agar, renewed every fortnight. For experimental purposes it was grown in a liquid medium. Two media were used: a vitamin B₆ free medium and a medium containing pyridoxal, the latter was prepared by adding 500 µg of pyridoxal (Merck) to each litre of the vitamin B₆-free medium. The vitamin B₆ free medium was similar to that developed by Bellamy & Gunsalus (1945), its composition is given in Table 1. The concentration of K₂HPO₄ in the medium was double that given by Bellamy & Gunsalus, this change approximately trebled the yield of tyrosine decarboxylase.

Table 1 *Medium used for growth of Streptococcus faecalis* R

(Quantities for 1 l of solution, adjusted to pH 7.2-7.3 before autoclaving)

Casam hydrolysate, dried, vitamin free	10 g
Anhydrous glucose	10 g
K ₂ HPO ₄	10 g
Sodium acetate (hydrated)	2 g
Salts B (Landy & Dicken, 1942)	5 ml
DL-Alanine	200 mg
L Cystine	200 mg
DL Tryptophan	100 mg
Thioglycolic acid	100 mg
L Tyrosine	100 mg
Adenine sulphate	5 mg
Guanine hydrochloride	5 mg
Uracil	5 mg
Nicotinic acid	5 mg
Calcium dextropantothenate	1 mg
Riboflavin	1 mg
Pteroylglutamic acid	44 µg
Biotin	1 µg

Before it was grown in either of the experimental media, the organism was subcultured through a liquid medium prepared by adding 0.1 µg of pyridoxal to 9 ml of the vitamin B₆ free medium. After inoculation from the stab culture, this medium was incubated for 24 hr at 37°. The cell suspension was then diluted 20 times with sterile distilled water, and each 9 ml. of experimental medium was inoculated with 0.1 ml. of the diluted suspension.

Washed suspensions of intact resting cells were prepared as follows. Tubes, each containing 9 ml. of medium, were inoculated and incubated for 24 hr at 37°. The cells were then centrifuged out and washed with 0.9% (w/v) NaCl, for the manometric experiments the cells from each tube were suspended in 1.5 ml. of distilled water.

Dried cells of the organism were prepared as follows. For each sample a flask containing about 400 ml of warmed medium (with or without added pyridoxal) was inoculated and incubated at 37°. In the absence of pyridoxal the highest yields of tyrosine apodecarboxylase were obtained after 20 hr incubation, in the presence of pyridoxal the best time of incubation was c. 14 hr. The flask was then cooled to 0°, the cells were centrifuged out, washed with ice-cold 0.9% (w/v) NaCl and suspended in a little ice-cold distilled water. The suspension was poured into c. 7 vol of ice-cold dry acetone, the cells were then filtered off on a

small Buchner funnel (using Whatman no. 50 paper), washed with small portions of ice-cold dry acetone, and finally dried *in vacuo* over H₂SO₄. About 250 mg of dried cells were usually obtained from each 400 ml of medium. They were stored *in vacuo* over H₂SO₄ at 0°.

Manometric procedure In all experiments Warburg manometers were used, with conical flasks with one side bulb. The side bulb of each flask contained 0.4 ml of substrate solution, except in substrate competition experiments, in each of which the side bulb of one flask contained 0.4 ml of a solution of the amino acid to be tested plus 0.4 ml. of 0.04M-L tyrosine suspension. The temperature of the bath was 28.5°. In the experiments with intact cells the gas phase was air, except in the studies with 2.5 dihydroxyphenylalanine when N₂ was used. In all experiments with dried cells the gas phase was N₂.

The contents of the main compartments of the manometer flasks depended on the cell preparation used. In the experiments with intact cells grown in the vitamin B₆ free medium, the main compartments contained 0.5 ml of cell suspension, 1.0 ml of 0.075M phthalate buffer (pH 5.0), 1.0 ml of pyridoxal hydrochloride solution (containing 10 µg of pyridoxal) and 0.1 ml of distilled water. Under these conditions the tyrosine apodecarboxylase in the cells is saturated with codecarboxylase formed within the cells from the added pyridoxal (Gunsalus & Bellamy, 1944). In these experiments the rate of evolution of CO₂ was low in the first few minutes after tipping but subsequently increased, and became steady after about 15 min.

In the experiments with dried cells grown in the vitamin B₆ free medium, the main compartment of each manometer flask contained 1 mg of dried cells (added as a suspension in distilled water), 0.2 ml of M acetate buffer (pH 5.5), 1.0 ml of pyridoxal hydrochloride solution (containing 10 µg of pyridoxal), 0.1 ml of adenosinetriphosphate (ATP) solution (prepared from a sample of the barium salt, given by Boots Pure Drug Co Ltd., and containing 1 mg of ATP), distilled water was added to make the total volume of liquid, including that in the side bulb, 3 ml. Under these conditions, the tyrosine apodecarboxylase in the dried cells is saturated with codecarboxylase formed from the added pyridoxal and ATP by an enzyme in the cells (Umbreit, Bellamy & Gunsalus, 1945). With dried cells, the rate of evolution of CO₂ became steady within 5 min. after tipping.

In the experiments with dried cells grown in the medium containing pyridoxal (500 µg/l), the main compartment of each flask contained 10 mg of dried cells (added as a suspension in distilled water), 0.2 ml of M acetate buffer (pH 5.5) and distilled water to make the volume 1.1 ml. The dried cells used for these experiments contained tyrosine decarboxylase already saturated with codecarboxylase. These cells will be referred to as 'complete' cells. In these experiments CO₂ was evolved at the maximum (steady) rate from the moment of tipping.

In every experiment, the steady rate of evolution of CO₂ was measured, and is given as *V* in the Tables, *V_T* represents the rate with 0.4 ml of 0.04M-L-tyrosine added as substrate.

RESULTS

Experiments with m-hydroxyphenylalanine The results of some typical experiments are given in Table 2. Intact cells of *Strep faecalis* R were

tested with two different concentrations of *m*-hydroxyphenylalanine with the lower concentration no CO₂ was evolved (Exp 1), with the higher concentration a small amount of CO₂ was apparently evolved, but it is doubtful whether this was outside the limits of experimental error (Exp 2) With dried cells, however, *m*-hydroxyphenylalanine was definitely decarboxylated, the rate of reaction

water, and the washings were added to the tube. The mixture (8 ml) was expected to contain 1 mg of *m*-hydroxyphenylethylamine/ml. It was heated for 5 min in boiling water and then centrifuged. Portions of the supernatant fluid were injected into the jugular vein of a spinal cat. Fig 1 is a record of the arterial blood pressure and shows the results of the injection of (a) 1.0 mg of synthetic *m*-hydroxy-

Table 2 Tyrosine decarboxylase of *Streptococcus faecalis* R, and *m* hydroxyphenylalanine

Exp no	Enzyme preparation	Conc of DL- <i>m</i> -hydroxyphenylalanine (M)	V (μl CO ₂ evolved/hr)	V _T (μl CO ₂ evolved/hr, tyrosine as substrate)	V/V _T
1	Intact vitamin B ₆ free cells + pyridoxal	0.0027	0	300	0
2	Intact vitamin B ₆ free cells + pyridoxal	0.014	10	490	0.02
3	Dried vitamin B ₆ free cells + pyridoxal + ATP	0.011	220	810	0.27
4	Dried 'complete' cells grown with pyridoxal	0.022	360	1150	0.31

was about 30% of that with tyrosine as substrate.

Substrate competition experiments suggested that tyrosine and *m*-hydroxyphenylalanine are both decarboxylated by the same enzyme. In one such experiment the rates of evolution of CO₂, in μl/hr, were with 0.011 M-DL-*m*-hydroxyphenylalanine, 220, with 0.0053 M-L-tyrosine, 810, with both amino-acids, 590.

The product of the decarboxylation of *m*-hydroxyphenylalanine is the corresponding amine, *m*-hydroxyphenylethylamine. This substance has sympathomimetic properties and raises the arterial blood pressure of the spinal cat (Barger & Dale, 1910; Blaschko, Holton & Sloane-Stanley, 1949). The liquid from manometer flasks in which *m*-hydroxyphenylalanine had been incubated with dried 'complete' cells of *Strep faecalis* R was, therefore, injected into spinal cats whose arterial blood pressure was recorded, its pressor action was compared with that of synthetic *m*-hydroxyphenylethylamine. In one such experiment four manometer flasks were set up, each containing 10 mg of dried 'complete' cells and 0.022 M-DL-*m*-hydroxyphenylalanine in a total volume of 1.5 ml. They were shaken in the bath for 2 hr after tipping, by the end of which time the evolution of CO₂ had almost ceased, the results of Exp 4 were obtained from one of these flasks. The total volume of CO₂ which had been evolved in all four flasks was 1220 μl. The retention of CO₂ under these conditions had been found to be about 6% in a parallel experiment with tyrosine as substrate, therefore, the evolution of 1220 μl of CO₂ corresponded to the formation of 1290 μl, equivalent to 8 mg, of *m*-hydroxyphenylethylamine. The liquid from all four manometer flasks was pipetted into a centrifuge tube, each flask was washed twice with 0.25 ml of distilled

phenylethylamine, (b) 1.0 ml of the supernatant fluid, and (c) 0.7 mg of synthetic *m*-hydroxyphenylethylamine. The injection of the supernatant fluid had an effect on the blood pressure similar to that of

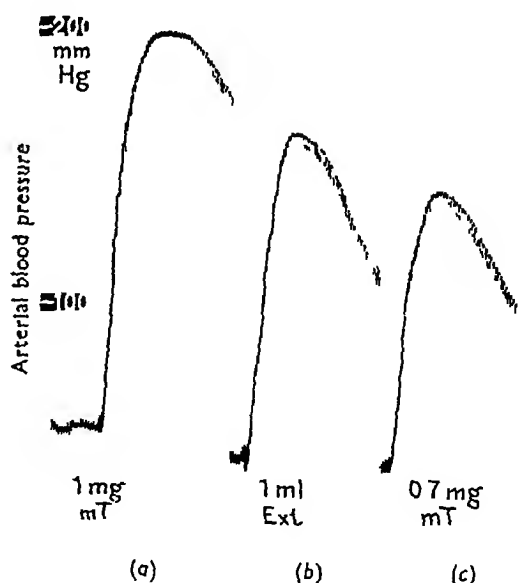


Fig 1 Pressor action of *m*-hydroxyphenylethylamine. Arterial blood pressure of spinal cat. Tracing shows effects of intravenous injection of (a) 1.0 mg of synthetic *m*-hydroxyphenylethylamine, (b) 1.0 ml of supernatant fluid from incubation of *m*-hydroxyphenylalanine with acetone dried preparation of *Strep faecalis* R, expected to contain 1.0 mg of *m*-hydroxyphenylethylamine, and (c) 0.7 mg of synthetic *m*-hydroxyphenylethylamine.

synthetic *m*-hydroxyphenylethylamine. The pressor response to 1.0 ml of the supernatant fluid was slightly less than that of 1.0 mg of the synthetic amine, this may have been due to the fall of the

initial blood-pressure level between injections (a) and (b). The pressor effect of 1.0 ml of the supernatant fluid was, however, greater than that of 0.7 mg of the amine. These results show that, within the limits of error of this method, the concentration of *m*-hydroxyphenylethylamine in the supernatant fluid, as determined by pharmacological assay, agreed with that calculated to be present from the results of the manometric experiment. The total volume of CO₂ formed in this experiment, 1290 μ l, was 43% of that which would have been formed by the complete decarboxylation of the *m*-hydroxyphenylalanine added. This suggests that only one of the two optical isomers, probably the L-form, was attacked.

Experiments with *o*-hydroxyphenylalanine. DL-*o*-Hydroxyphenylalanine (0.0107 M) was incubated with dried vitamin B₆-free cells plus pyridoxal and ATP. CO₂ was evolved at a rate of c. 25 μ l/hr, with 0.0053 M-L tyrosine as substrate, the rate of evolution of CO₂ was 810 μ l/hr. In a substrate competition experiment done at the same time, the rate of evolution of CO₂ with both substrates was 775 μ l/hr, which represented a decrease of 4% in the rate of decarboxylation of tyrosine; in another, similar, experiment the decrease was 10%. These decreases were probably not significant.

Experiments with 2,5-dihydroxyphenylalanine. The results of some typical experiments are given in Table 3. Intact cells of *Strep. faecalis* R did not decarboxylate 2,5-dihydroxyphenylalanine at all, but in some of the experiments with dried cells CO₂ was apparently evolved, but very slowly.

decreased the rate of decarboxylation of the same amount of L-tyrosine by only 8% (from 370 to 340 μ l of CO₂/hr).

DISCUSSION

The results of the experiments with the isomers of tyrosine have shown that the rates of decarboxylation of these amino-acids by dried preparations of *Strep. faecalis* R decrease in the order tyrosine, *m*-hydroxyphenylalanine, *o*-hydroxyphenylalanine. The meta compound was decarboxylated by acetone-dried preparations of the organism at about 30% of the rate observed with tyrosine, *m*-hydroxyphenylethylamine was formed in this reaction, as shown by its effect on the arterial blood pressure of the spinal cat. Substrate competition experiments suggested that both tyrosine and *m*-hydroxyphenylalanine were decarboxylated by the same enzyme. With *o*-hydroxyphenylalanine CO₂ was evolved so slowly that it is doubtful whether this amino-acid was decarboxylated at all. In substrate competition experiments it caused negligible decreases in the rate of decarboxylation of tyrosine, its affinity for tyrosine decarboxylase must, therefore, be very low.

In the experiments with *m*-hydroxyphenylalanine no CO₂ was evolved in the presence of washed suspensions of intact resting cells of the organism, as a result of this observation it was stated that the tyrosine decarboxylase of *Strep. faecalis* does not attack *m*-hydroxyphenylalanine (Blaschko & Sloane Stanley, 1948). But the experi-

Table 3 Tyrosine decarboxylase of *Streptococcus faecalis* R, and 2,5-dihydroxyphenylalanine

Exp. no.	Enzyme preparation	Conc. of 2,5-dihydroxyphenylalanine (M)	V (μ l CO ₂ evolved/hr)	V _T (μ l CO ₂ evolved/hr, tyrosine as substrate)	V/V _T
5	Intact vitamin B ₆ free cells + pyridoxal	0.011 (DL)	0	350	0
6	Intact vitamin B ₆ free cells + pyridoxal	0.0053 (L)	0	380	0
7	Dried vitamin B ₆ free cells + pyridoxal + ATP	0.0107 (DL)	20	810	0.025
8	Dried vitamin B ₆ free cells + pyridoxal + ATP	0.0053 (L)	0	370	0
9	Dried 'complete' cells (grown with pyridoxal)	0.0107 (L)	15	490	0.03

In order to find out whether tyrosine decarboxylase can combine with 2,5-dihydroxyphenylalanine, substrate competition experiments were done. In Exp. 7, a mixture of 0.4 ml of 0.08 M-DL-2,5-dihydroxyphenylalanine plus 0.4 ml of 0.04 M-L-tyrosine was added as substrate, the rate of evolution of CO₂ was 590 μ l/hr, thus the addition of 2,5-dihydroxyphenylalanine had decreased the rate of decarboxylation of tyrosine by 27% (V_T = 810). In another experiment the addition of 0.4 ml of 0.04 M-L-2,5-dihydroxyphenylalanine

ments described in this paper have shown that although this amino-acid is not decarboxylated by intact cells of *Strep. faecalis* R, it is attacked by acetone-dried preparations of the organism. This difference is interesting for it suggests that the undamaged cell membranes of the intact cells are impermeable to *m*-hydroxyphenylalanine, although they are freely permeable to the isomeric tyrosine.

It is not certain whether 2,5-dihydroxyphenylalanine is a substrate of tyrosine decarboxylase, or

can combine with the enzyme. It was apparently decarboxylated very slowly by acetone-dried preparations of *Strep faecalis* R, and it caused slight decreases in the rates of decarboxylation of tyrosine by these preparations, but these effects may have been within the limits of experimental error.

As a result of the experiments of Epps (1944) it is evident that the tyrosine decarboxylase of *Strep faecalis* attacks both tyrosine and 3,4-dihydroxyphenylalanine. The experiments described in the present paper have not been done with pure preparations of the enzyme, but the evidence presented suggests that *m*-hydroxyphenylalanine is in fact a substrate of tyrosine decarboxylase. It is evident that the replacement of the para-hydroxyl group of tyrosine by a hydroxyl group in the meta position relative to the side chain produces a substance which is still decarboxylated by *Strep faecalis*, on the other hand, our experiments with *o*-hydroxyphenylalanine have shown that the rate of decarboxylation of this compound by tyrosine decarboxylase is very low.

SUMMARY

1. Acetone-dried preparations of *Strep faecalis* R decarboxylate *m*-hydroxyphenylalanine at about 30% of the rate at which they decarboxylate tyrosine. The reaction appears to be specific for the L form of *m*-hydroxyphenylalanine, which is decarboxylated quantitatively to *m*-hydroxyphenylethylamine. Washed suspensions of intact resting cells of the organism have no detectable action on *m*-hydroxyphenylalanine.

2. Dried preparations of *Strep faecalis* R do not decarboxylate *o*-hydroxyphenylalanine at a significant rate, nor has this amino-acid any detectable affinity for tyrosine decarboxylase.

3. Neither dried preparations nor intact cells of *Strep faecalis* R have any significant action on 2,5-dihydroxyphenylalanine.

I wish to thank Dr H. Blaschko for his help and encouragement, and Mrs P. Holton for carrying out the pharmacological assays. I am grateful to the Medical Research Council for a Research Training Grant.

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The Effects of Applied Pressure on Secretion by Isolated Amphibian Gastric Mucosa

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Ludwig (1851) has shown that secretion by the sub-maxillary gland can occur against a pressure greater than the arterial blood pressure, but no evidence is available about the relation between pressure and secretion in the stomach. The apparatus described in this paper was developed to study simultaneously the rates of fluid secretion and respiration by isolated gastric mucosa. The results show that the secretion of hydrochloric acid can occur against pressure. This supports the view that the hydrostatic pressure of the blood is not an essential part of the mechanism of secretion of the solution of hydrochloric acid by the oxyntic cell.

Apparatus

EXPERIMENTAL

The apparatus used is shown in Figs 1 and 2. It consists of a manometer *A*, a vessel *B*, and a hollow stopper *S*. *A* is a constant pressure manometer (modified from Dixon, 1943). Like a Warburg manometer it consists of a U tube provided with a rubber reservoir *R*₁ containing modified Brodie solution. A graduated 0.5 ml pipette *P* is attached to its right hand limb, a rubber reservoir *R*₂ containing mercury is attached to the end of the pipette. The vessel *B* is a conical cup (volume c. 27 ml.) with a central ground socket *J*₁ and two side arms *C*₁ and *C*₂. The vessel is attached to the manometer by the ground joint *J*₂ on the side arm *C*₁. The side arm *C*₂, which is not shown in Fig. 2, has a tap

stopper. Instead of the usual centre well a side well W is fused to the wall of the vessel. The stopper S fits into the socket J_1 . The hollow base of S dips into the nutrient saline in B , and communicates with a graduated horizontal capillary side tube G which can be closed by tap T . The stopper S is in turn closed by a tap stopper V , which must have a wide hole to allow easy flow of the excess liquid paraffin (see Methods).

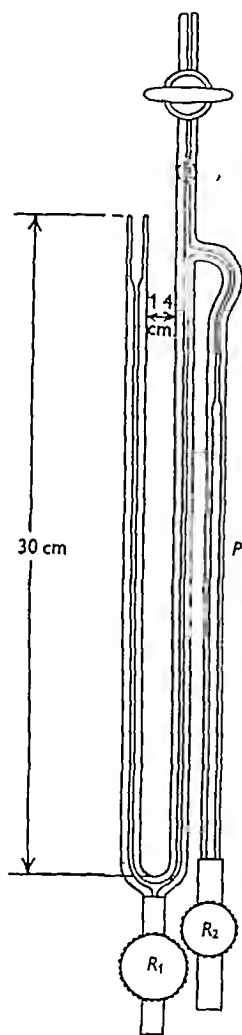


Fig 1 Constant pressure manometer. R_1 , reservoir for Brodie solution, R_2 , reservoir for mercury, outer diameter of tubing 0.75 cm, P , mercury pipette, bore 0.18 cm, capacity 0.5 ml, graduation 1 division = $5 \mu\text{l}$ (length 20 cm). U-tube graduated in mm (bore 0.11 cm).

Theory of the apparatus

Dixon (1943) described a differential constant pressure manometer which had some similarities to the present apparatus, but the theory of the system when used for CO_2 measurements was not given.

Case 1 Gas space filled with 100% O_2 , CO_2 absorbed by NaOH. The amount of O_2 absorbed is given by the readings of pipette P , multiplied by a factor correcting it to N.T.P.

Case 2 Gas space filled with 5% CO_2 + 95% N_2 . CO_2 is evolved and the partial pressures of both CO_2 and N_2 change. Since the solubility of CO_2 is high ($\alpha = 0.74$ at 25°), the amount dissolved in the liquid changes appreciably. The solubility of N_2 , however, is small ($\alpha = 0.015$ at 25°), and

since the partial pressure changes during an experiment are not more than 0.3%, the changes in the amount of N_2 dissolved in the liquid can be safely neglected. The simplified theory of the system for measuring the anaerobic glycolysis is given below.

Case 3 Gas space filled with 5% CO_2 + 95% O_2 . O_2 is absorbed and respiratory CO_2 is evolved. In a non-secreting mucosa the CO_2 output nearly balances the O_2 uptake so that the manometric readings show a very small resultant uptake of gas. In a secreting mucosa 1 mol of CO_2 is absorbed to form a bicarbonate ion for each molecule of HCl produced. The increase in the metabolic rate of the tissue during secretion is usually about 30%, and, as was shown by Davies (1948), no significant change occurs in the overall uptake as measured on Warburg manometers. The

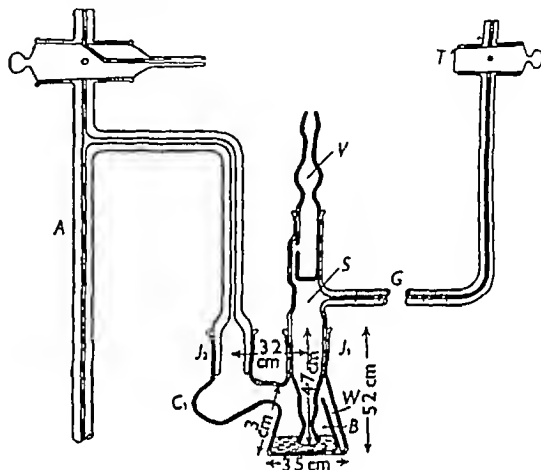


Fig 2 Vessel B , hollow stopper S , with graduated side tube G , and part of manometer A . Capacity of B , 25 ml, capacity of side arms C_1 and C_2 (C_2 is not shown), 1 ml. Outer diameter of G , 0.6 cm, bore 0.1 cm, length 15 cm, graduation 1 division = $1 \mu\text{l}$. The mucosa is tied to the base of S (diameter 0.9 cm).

rate of uptake of the CO_2 absorbed by the mucosa during secretion of acid can, therefore, be used to estimate the rate of secretion of HCl (see Davies, 1948). The solubility of O_2 is low ($\alpha = 0.028$ at 25°), and so the simplified theory of the system, when used for measuring the uptake of CO_2 due to acid secretion, is the same as in Case 2.

Let x = the amount of gas evolved (μl at N.T.P.),

v = the observed change in the readings of the mercury pipette (μl),

V_0 = the volume of the gas space in the vessel and manometer (μl),

V_F = the volume of the liquid in the vessel (μl),

T = the absolute temperature,

P = the initial pressure in the vessel (i.e. the barometric pressure \pm the pressure maintained in the manometer, mm Brodie solution),

P_0 = the normal pressure (10,000 mm Brodie solution),

p = the vapour pressure of water at temperature T (mm Brodie solution),

α = Bunsen's absorption coefficient (expressed as ml. gas/ml. liquid when the partial pressure of the gas is equal to P_0) at N.T.P.

c = the initial percentage of the gas under consideration in the gas mixture of the cylinder, and

c' = the final percentage of this gas in the gas space

Case 1 When 100% O_2 is used and CO_2 is absorbed by NaOH

$$x = v \frac{273}{T} \frac{P-p}{P_0},$$

$$x = v l_1$$

Cases 2 and 3 Gas space filled with 5% CO_2 + 95% N_2 , or 5% CO_2 + 95% O_2 . Since the total amount of gas in the apparatus equals amount of gas in gas phase plus amount of gas in solution, the initial amount of gas equals

$$V_g \frac{c}{100} \frac{273}{T} \frac{P-p}{P_0} + V_F \alpha \frac{c}{100} \frac{P-p}{P_0}, \quad (i)$$

and the final amount of gas equals

$$(V_g + v) \frac{c'}{100} \frac{273}{T} \frac{P-p}{P_0} + V_F \alpha \frac{c'}{100} \frac{P-p}{P_0} \quad (ii)$$

Hence by subtraction,

$$x = \frac{c'}{100} \frac{P-p}{P_0} \left[(V_g + v) \frac{273}{T} + V_F \alpha \right] - \frac{c}{100} \frac{P-p}{P_0} \left[V_g \frac{273}{T} + V_F \alpha \right] \quad (iii)$$

Since v = the increase in the volume of the gas space of the manometer at constant pressure due to the evolution of gas,

$$v = \frac{c'}{100} (V_g + v) - \frac{c}{100} V_g, \quad (iv)$$

and

$$c' = 100 \frac{v + \frac{c}{100} V_g}{V_g + v} \quad (v)$$

By substitution of (v) in (iii),

$$x = v \frac{273}{T} \frac{P-p}{P_0} + v \frac{V_F \alpha}{V_g + v} \frac{P-p}{P_0} \left(1 - \frac{c}{100} \right) \quad (vi)$$

v is small compared with V_g and an error of less than 0.2% is made if v is neglected in the denominator of the second term of (vi) and the equation used in the form

$$x = v \left[\frac{273}{T} \frac{P-p}{P_0} + \frac{V_F \alpha}{V_g} \frac{P-p}{P_0} \left(1 - \frac{c}{100} \right) \right], \quad (vii)$$

or $x = v l_2$

In most of our experiments in which gas mixtures containing 5% CO_2 were used, l_2 was 1.02–1.04 depending on V_F .

The constant pressure manometers were compared with the standard Warburg manometers. The evolution of CO_2 from $NaHCO_3$ solution by adding a known amount of standard acid solution and the uptake of O_2 by a yeast suspension agreed in both types of apparatus.

Saline media

The media were prepared from the stock solutions used for making the physiological saline of Krebs & Henseleit (1932). (1) a basal saline isotonic with frog serum (modified from Gray, Adkison & Zelle, 1940), prepared by adding to 2000 ml of doubly distilled water 79 ml $NaCl$ (18%), 81 ml KCl (1.15%), 34.7 ml $CaCl_2$ (0.11M), 12 ml KH_2PO_4 (0.2M), 15.8 ml $MgSO_4 \cdot 7H_2O$ (3.82%), 47.3 ml $NaHCO_3$ (1.3%), (2) 0.12M $NaHCO_3$ solution, (3) 0.080M-phosphate

buffer, pH 7.4. During experiments in 100% O_2 a phosphate saline was used which was prepared by adding 1 vol of solution (3) to 10 vol. of solution (1). CO_2 was absorbed by NaOH in the side well of the flask. For experiments in 5% CO_2 and 95% O_2 a bicarbonate saline was prepared by adding 16 vol of solution (2) to 100 vol of solution (1). This bicarbonate saline was also used in anaerobic experiments in 5% CO_2 and 95% N_2 . Traces of O_2 in the gas mixture were removed by a stick of yellow phosphorus in the side well. All nutrient salines contained 0.2% glucose.

Material

Frogs (*Rana temporaria temporaria* L.) and toads (*Bufo bufo* L.) were used. They were captured locally and housed in an outdoor froggery under natural conditions. An excess of worms and insects was provided for food. Similar results were obtained with both species, but toad gastric mucosa is less fragile and was preferred. The animal was pithed and the stomach isolated and washed with frog saline. The muscle layer was removed and the tube of mucosa cut open along the lesser curvature (Davies, 1948). The open sheet was then tied, with the secretory side upwards, to the open base of the stopper S and the excess of tissue cut away. In early experiments the mucosa was stretched tightly over the end of the stopper, but was found to tear easily under the stress of applied pressure. This was prevented by laying the mucosa loosely over the end of the stopper before tying, it could then withstand pressures much greater than 300 mm (Fig. 3), but large

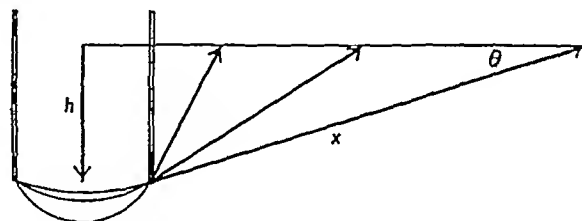


Fig. 3 Effect of tightness of membrane on stress. Let h = total pressure on secretory side of mucosa, x = resultant stress in mucosa, θ = angle of stress vector to horizontal. From the triangle of forces $h/x = \sin \theta$. When $\theta = 5^\circ$, $x = 11.5 h$, when $\theta = 30^\circ$, $x = 2.0 h$.

fluctuations occurred in the readings. The continuous slight adjustments of the pressure in the manometer caused visible contractions of the muscularis mucosae. It was found, however, that 10^{-3} M-atropine in the nutrient saline stopped these movements, but did not prevent good secretory responses to histamine. This concentration of atropine was therefore used in all later experiments.

Methods

The secretory surface of the mucosa was covered with 0.2 ml. of 0.12M- $NaCl$ pipetted into stopper S . The stopper and part of the graduated side tube G were then filled with liquid paraffin. Tap T at the end of G was shut and the tap stopper V was inserted gently to avoid stretching the mucosa. S was then placed into the main compartment of vessel B which contained sufficient nutrient saline (4–5 ml.) to cover the mucosa. The unit was attached to the manometer, filled with an appropriate gas mixture, placed in a

water bath at 25° and shaken in a horizontal plane. An identical unit in which the mucosa was replaced by a rubber or plastic membrane was used as a thermobarometer. After equilibration the pressure inside the manometer was adjusted to the desired value, stopper *V* turned off and tap *T* opened.

The pressure acting on the mucosa was the difference of two components: (a) the pressure of the gas in the manometer, regulated by means of the mercury in *P* (Fig. 1), (b) the hydrostatic pressure of the column of liquid in stopper *S* less the hydrostatic pressure of the nutrient medium in the cup *B*. When (a) balanced (b) the resultant pressure was zero. When (a) was greater than (b), pressure was exerted in the direction of normal secretion (from the nutrient to the secretory side of the mucosa), the resultant pressure (a)–(b) was positive and was prefixed with a positive sign. When (a) was less than (b) the resultant pressure was opposed to the direction of normal secretion and was given a negative sign. All pressures recorded in this paper are in mm. Brodie solution (Dixon, 1943). Since the gas exchanges of the tissue and the transport of liquid across the mucosa altered the pressure in the manometer, the mercury in *P* had to be adjusted continuously in order to keep the pressure on the mucosa constant at any desired value. This value could be altered at will several times during an experiment. The possible range of pressures was from –300 to +300.

The mucosa secreted either spontaneously, or following the addition from the side arm of histamine to $5 \times 10^{-5} M$. These secretions displaced the liquid paraffin, the movement of which along the graduated capillary tube *G* was measured. At the same time measurements of the gaseous exchanges were made with pipette *P*.

Three types of experiment were performed: (1) The rates of respiration (Q_{O_2}) and fluid secretion (q_{H_2O}) were measured in phosphate saline gassed with 100% O_2 , (2) the rate of acid secretion (Q_{HCl}) and the q_{H_2O} in bicarbonate saline gassed with 5% CO_2 + 95% O_2 , (3) the rate of anaerobic glycolysis ($Q_{CO_2}^N$) and the q_{H_2O} in bicarbonate saline gassed with 5% CO_2 + 95% N_2 . For each set of measurements one mucosa was used.

After the experiment the mucosa was removed in two portions by cutting round the silk ligature with a sharp razor, washed in distilled water and dried overnight at 110°. The dry weight of the circular portion was used in the calculation of the Q_{HCl} , and q_{H_2O} , while the Q_{O_2} and $Q_{CO_2}^N$ were calculated using the sum of the dry weight of the two portions. The values of the Q_{HCl} , Q_{O_2} and $Q_{CO_2}^N$ are in μl /mg dry wt./hr (HCl being considered as a gas at $N.T.P.$), the q_{H_2O} is defined as μl transported fluid/mg dry wt./hr.

Calculation of results

Since the secretion of liquid into stopper *S* during any period resulted in a loss of liquid from the cup *B*, allowance had to be made for this reduction of liquid volume when calculating the gas exchanges. Thus, when the change of volume in the mercury pipette *P* was $-10 \mu l/15 \text{ min}$, while the increase in the volume of liquid in the graduated capillary tube *G* was $+2 \mu l/15 \text{ min}$, the uptake of gas was actually $-10 + 2 = -8 \mu l/15 \text{ min}$. This was multiplied by a constant which depended on the gas used, to give the volume of gas at $N.T.P.$ (see Theory of the apparatus). Specimen protocols are given at the end of the paper.

RESULTS

Water transport and pressure. Application of pressures of approximately $\pm 50 \text{ mm}$ did not force liquid across a membrane of aerobic gastric mucosa which was not secreting. Mucosae secreting either spontaneously or after stimulation with histamine transported liquid from the nutrient to the secretory side only. The rate was not influenced by the direction or magnitude of the applied pressures within the limits of about $\pm 50 \text{ mm}$ (Fig. 4). The average maximum q_{H_2O} calculated for twenty-four secreting

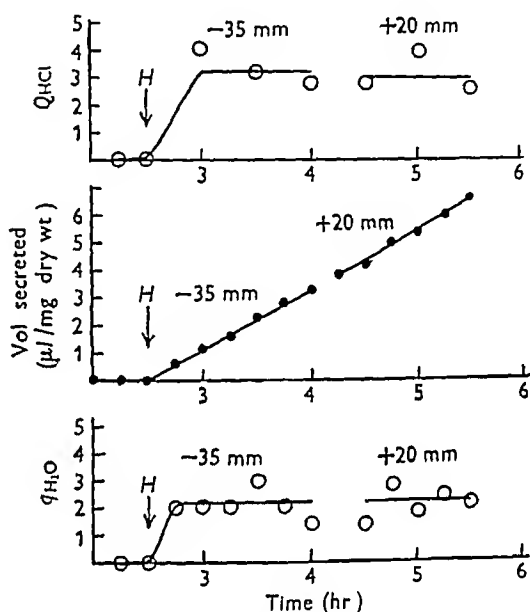


Fig. 4. Effect of pressure on Q_{HCl} and Q_{O_2} . Histamine was added to a concentration of $5 \times 10^{-5} M$ at arrows marked *H*. The applied pressures are given in mm. Brodie solution.

mucosae was between 1.0 and 4.0, average 2.3, standard deviation 0.9. The average maximum Q_{HCl} in ten secreting mucosae was from 2.8 to 7.0, average 4.8, standard deviation 1.3. The average maximum Q_{O_2} in twelve secreting mucosae was from 1.9 to 4.8, average 3.2, standard deviation 1.0. The pressures in these experiments ranged from –80 to +50 mm. The highest opposing pressure so far used was –120 mm and acid was secreted by the mucosa. The figure of 18 μl secretion/mg dry wt./hr stated by Davies & Turner (1948) has been found to be in error because of a leak due to a fault in tap *V* of the stopper *S*. This fault has now been corrected. In some experiments neutral red was added to the nutrient saline to a final concentration of $10^{-4} M$. It was transported from the nutrient to the secretory side against opposing pressures (cf. Bradford & Davies, 1948). No water transport or HCl secretion occurred in mucosae under anaerobic conditions when the resultant pressure was zero. When the

Table 1 *Effect of pressure on transport of fluid across frog gastric mucosa*

(+ Indicates direction of natural secretion, i.e. from nutrient to secretory side, - the direction opposed to natural secretion)

Mucosa no	Date	Gas mixture	Experimental time	Resultant pressure (mm. Brodie solution)	q_{H_2O} (μ l/mg dry wt/hr)	Remarks
1	1 x 47	5% CO ₂ +95% N ₂	0 30-1 45	-10	-0 4	$Q_{CO_2}^N=1 6$
			1 50-3 00	-30	-0 9	
			3 05-4 00	+20	+0 9	
			4 05-5 00	+40	+1 35	
2	21 vi 48	5% CO ₂ +95% N ₂	1 58-2 23	-30	-5 2	$Q_{CO_2}^N=2 0$
			2 28-3 18	0	0	
3	9 vi 48	100% O ₂	1 38-3 08	-35	+0 92	Histamine added to 5 \times 10 ⁻⁵ M at 0 hr 53 min
			3 23-4 23	-65	+0 46	
4	15 viii 47	5% CO ₂ +95% O ₂	1 00-1 30	-42	+1 64	Histamine added to 5 \times 10 ⁻⁵ M at 1 00 hr
			1 40-3 05	-12	+1 0	
			3 10-4 40	+18	+1 64	
			4 50-5 30	+33	+1 36	

pressure was increased above, or reduced below, zero a neutral fluid was forced across the mucosa in the direction of the applied pressure. Typical results are given in Table 1.

Fig. 5 gives the results of two experiments done simultaneously on two pieces, A and B, of one large mucosa which had been divided along the greater and lesser curvatures. The two portions of mucosa from the corpus were tied over the end of two different stoppers. A was used to measure the q_{H_2O} and Q_{O_2} . The nutrient solution was phosphate saline gassed with 100% O₂. CO₂ was absorbed by sodium hydroxide in the side well. B was placed in bicarbonate saline gassed with 5% CO₂ and 95% O₂ and measurements made of the q_{H_2O} and Q_{HCl} . As described on p. 378, in a non-secreting mucosa the CO₂ output nearly balances the O₂ uptake, but in a secreting mucosa one molecule of CO₂ is absorbed to form bicarbonate for each molecule of HCl produced. The rate of uptake of this CO₂ can, therefore, be used to estimate the rate of secretion of HCl. A comparison of the two pieces of mucosa showed that the Q_{O_2} , Q_{HCl} and q_{H_2O} increased simultaneously after stimulation with 5 \times 10⁻⁵ M-histamine. Both A and B secreted against a pressure of -30 maintained throughout the experiments. The molarity of the HCl in the secretion was calculated from the q_{H_2O} and Q_{HCl} , and was found to be between 0.09 and 0.10.

The time lapse between mixing and the response to histamine varied from 1 min. to 2 hr. and the responses were of varying intensity and duration (Davies, 1948). When the secretory phase was over, the volume of the secretion did not increase and the q_{H_2O} and Q_{HCl} fell to zero. The Q_{O_2} returned to its basal value. It was also found that mucosae that had stopped secreting were not influenced by pressures of the order of \pm 50 mm. of water.

A difficulty was encountered during experiments

with actively acid secreting mucosae in phosphate saline. These often ulcerated because there were insufficient supplies of CO₂ to neutralize the alkali.

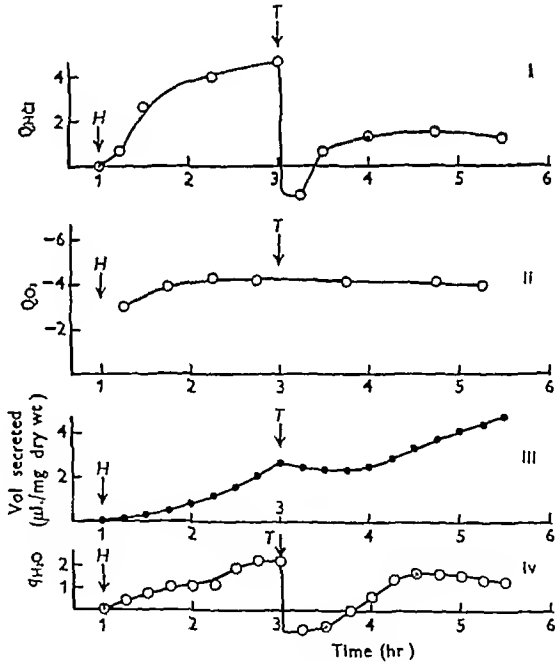


Fig. 5 Effect of histamine and thiocyanate on Q_{HCl} , Q_{O_2} and q_{H_2O} . The curves II, III and IV result from an experiment in 100% O₂ on the anterior portion A, curve I from an experiment in 5% CO₂ and 95% O₂ on the posterior portion B, of the same gastric mucosa. The secretion curves of B were similar to those of A (III and IV) and are not given. Both A and B were secreting against a resultant pressure of -30 mm. Brodie solution. Histamine was added to a concentration of 5 \times 10⁻⁵ M at arrows marked H, thiocyanate to a concentration of 10⁻² M at arrows marked T.

formed in the oxyntic cell concomitantly with the acid (cf Davies & Longmuir, 1948).

Effect of thiocyanate Thiocyanate is known to inhibit acid secretion *in vivo* (Davenport, 1940) and *in vitro* (Crane, Davies & Longmuir, 1946). It was observed that 10^{-2} M-thiocyanate also reduced the volume of the secretions (Fig 5, 1 and III). Secretion of acid was usually stopped, for 30–45 min. The mucosa appeared to lose its relative impermeability in either direction. With opposing pressures some water and acid passed back through the mucosa into the cup, and this resulted in an output of CO_2 if bicarbonate saline was used as the medium (Fig 5, 1).

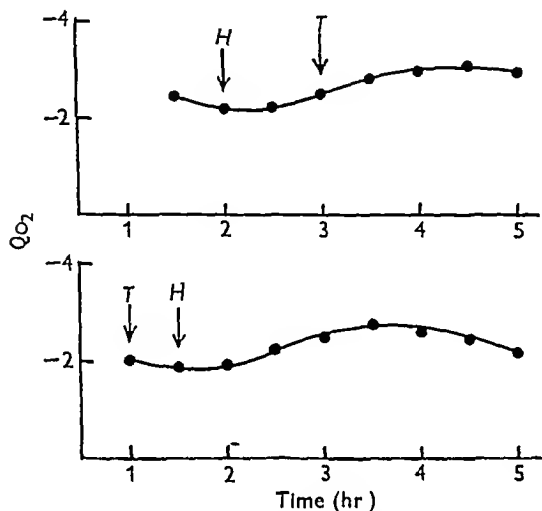


Fig 6 Effect of histamine and thiocyanate on Q_{O_2} . Sheets of frog gastric mucosa were incubated at 25° in phosphate saline gassed with 100% O_2 in Warburg cups with 2 side arms. Histamine was added to a concentration of 5×10^{-5} M at arrows marked H, thiocyanate to a concentration of 10^{-2} M at arrows marked T.

This movement of liquid and CO_2 output were greater for greater opposing pressures. After 30–45 min, however, the mucosa recovered and resumed secretion of water and acid, though at lower rates. Neither the basal Q_{O_2} , nor the increase in the Q_{O_2} following histamine stimulation, was affected by 10^{-2} M-thiocyanate (Fig 6), whereas the Q_{HCl} was inhibited by 60–95% of its value during secretion.

DISCUSSION

Pressure and secretion Ludwig (1851) inserted a cannula into the duct of the submaxillary gland and showed that the secretory pressure could exceed the arterial blood pressure. The apparatus described in this paper makes it possible to study the relation between secretion and pressure in isolated sheets of tissue as opposed to glands with large ducts. This type of experiment is not usually possible in the intact mammal because pressures applied to the gastric mucosa, for instance, cause collapse of the blood capillaries and the tissue stops secreting, probably because of the resulting anoxia.

The experiments described in this paper show that

under aerobic conditions secretion by isolated amphibian gastric mucosa can occur against pressure, and that the rate of secretion is not increased by pressures applied in the direction of the natural secretion. This result bears on the statements by Davenport & Fisher (1940) that 'since the osmotic pressures of blood and gastric juice are the same the mol fraction of water in each is the same. Consequently, no reversible work is done when water passes from the blood to the secretion, and no reversible work is expended. A very small amount of irreversible work is done on the water in overcoming viscosity when water moves at a finite rate. The energy necessary to perform this work is doubtless derived from the difference in hydrostatic pressure between the blood and the gastric juice', and by Gray (1943) that 'it is conceivable that the filtration pressure of the blood does supply the energy required to move water against the mechanical resistance offered by the tissues and cell membranes'. Since secretion can occur, not only in the absence of blood pressure but even against resultant pressures, it is now certain that blood pressure is not necessary to move water through the tissues during acid secretion by gastric mucosa.

Under anaerobic conditions applied pressures caused a seepage of water through the mucosa in the direction of the pressures. This did not occur in the presence of O_2 , and shows that aerobic metabolism maintains the membrane in a water-tight condition.

The effects of thiocyanate The thiocyanate ion is known to inhibit gastric secretion (Davenport, 1940), and, although it can inhibit carbonic anhydrase, the reduction of the Q_{HCl} must depend on other processes (Feldberg, Keilm & Mann, 1940). Since 10^{-2} M-thiocyanate caused 60–95% inhibition of the Q_{HCl} , but did not inhibit the increase in the Q_{O_2} following histamine, nor prevent this increase when added before histamine, it seems possible that thiocyanate inhibits H^+ ion production by interfering with the metabolic chain at some point past that at which histamine acts.

The immediate effect of the thiocyanate was to allow the mucosa to let through water in response to applied pressures in a similar way to that under anaerobic conditions. If there was a pressure opposing secretion then some of the hydrochloric acid which had been secreted earlier could seep back through the mucosa. This early phase after the addition of 10^{-2} M-thiocyanate ended in 30–45 min, after which secretion continued at lower but steady rates.

It has not so far been possible to separate experimentally the secretion of acid and water by oxyntic cells, and the evidence at present available supports the view that there is a very close connexion between the mechanisms by which the acid and the water of the histamine-stimulated gastric juice are secreted by oxyntic cells.

SPECIMEN PROTOCOLS

(See calculation of results, p 380)

(1) *Respiration and fluid secretion*

19 April 1948 Nutrient medium phosphate saline containing 0.2% glucose and 0.001 M-atropine Gas 100% O₂, CO₂ absorbed by NaOH Histamine added from side arm at 90 min (concentration in medium after mixing 5×10^{-5} M) Temperature of bath 25.0° Barometric pressure 751 mm Hg Resultant pressure applied to mucosa -15 mm Brodie solution Dry weight of mucosa 18.5 mg, dry weight of secreting portion 11.7 mg

Time (min)	Change of manometer readings (μ l)	Change of side tube readings (μ l)	Change of gas volume		Q_{O_2} (μ l/mg dry wt/hr)	q_{H_2O} (μ l/mg dry wt/hr)
			At experimental temp and pressure (μ l)	At N T P (μ l)		
0 (in bath)						
30-60	-14	+2	-12	-10	-1.1	+0.3
90	-15	+2	-13	-11	-1.2	+0.3
90 (histamine added)						
120	-20	+4	-16	-14	-1.5	+0.7
150	-26	+7	-19	-17	-1.8	+1.2
180	-25	+8	-17	-15	-1.6	+1.4

(2) *Hydrochloric acid and fluid secretion*

25 June 1948 Nutrient medium bicarbonate saline containing 0.2% glucose and 0.001 M atropine Gas 5% CO₂ + 95% O₂ Histamine added from side arm at 45 min (concentration in medium after mixing 5×10^{-5} M) Temperature of bath 25.0° Barometric pressure 760 mm Hg Resultant pressure applied to mucosa -35 mm Brodie solution Dry weight of mucosa 7.9 mg, dry weight of secreting portion 5.6 mg $k_{CO_2} = 1.04$

Time (min)	Change of manometer readings (μ l)	Change of side tube readings (μ l)	Change of gas volume at experimental temp and pressure (μ l)	Change of gas volume due to HCl secretion		Q_{HCl} (μ l/mg dry wt/hr)	q_{H_2O} (μ l/mg dry wt/hr)
				At experi- mental temp and pressure (μ l)	At N T P (μ l)		
0 (in bath)							
30-45	-1.5	+0.5	-1*	0	0	0	+0.4
45 (histamine added)							
60	-9	+3	-6	-5	-5.2	+3.7	+2.2
75	-8	+3	-5	-4	-4.2	+3.0	+2.2
90	-10	+4	-6	-5	-5.2	+3.7	+2.9
105	-8	+3	-5	-4	-4.2	+3.0	+2.2
120	-7	+2	-5	-4	-4.2	+3.0	+1.4

* The small uptake of gas before addition of histamine is the resultant of the respiratory exchanges of the mucosa, and is subtracted from the volume changes occurring during each period after the addition of histamine, to give the changes of gas volume due to HCl secretion (see Theory of apparatus, Case 3)

(3) *Anaerobic glycolysis and fluid transport*

21 June 1948 Nutrient medium bicarbonate saline containing 0.2% glucose Gas 5% CO₂ + 95% N₂ Temperature of bath 25.0° Barometric pressure 759 mm Hg Resultant pressure applied to mucosa +10 mm Brodie solution Dry weight of mucosa 6.5 mg, dry weight of secreting portion 3.8 mg $k_{CO_2} = 1.04$

Time (min)	Change of manometer readings (μ l)	Change of side tube readings (μ l)	Change of gas volume		Q_{CO_2} (μ l/mg dry wt/hr)	q_{H_2O} (μ l/mg dry wt/hr)
			At experimental temp and pressure (μ l)	At N T P (μ l)		
0 (in bath)						
60-75	+3	+0.5	+3.5	+3.6	+2.2	+0.5
90	+5	+0.5	+5.5	+5.7	+3.5	+0.5
105	+4	+0.5	+4.5	+4.7	+2.9	+0.5

SUMMARY

1 An apparatus is described which has been used to study simultaneously the rates of fluid secretion and of respiration by isolated gastric mucosa. It consists of a constant pressure manometer, a modified Warburg cup and a stopper with a graduated capillary side tube (Figs 1 and 2). External pressure can be applied to the mucosa and be kept constant or varied at will.

2 Secretion occurred against opposing pressures of at least -120 mm Brodie solution, and was, within the limits of ± 50 mm Brodie solution, independent of the magnitude and direction of the applied pressures. Neutral red was transported across the mucosa against such pressures.

3 On stimulation with 5×10^{-5} M-histamine the rates of secretion and of respiration increased concomitantly.

4 In anaerobic experiments neutral fluid was

forced across the mucosa in the direction of the applied pressure. This did not occur with non-secreting mucosae under aerobic conditions.

5 Thiocyanate (10^{-2} M) did not affect the basal Q_{O_2} or the increase of the Q_{O_2} which followed stimulation by 5×10^{-5} M-histamine, but strongly inhibited the rate of hydrochloric acid secretion. Thiocyanate often caused passage of water across the mucosa in the direction of the external pressure, for up to 1 hr before secretion of acid and liquid was resumed at lower but steady rates.

6 These results show that the energy for the movement of water across acid-secreting gastric mucosa is derived not from the difference in hydrostatic pressure between the blood and the gastric juice, but from the aerobic metabolism of the oxyntic cells.

The authors wish to thank Prof H A Krebs, F R S, for help and criticism.

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Globulin Complexes with Oestrogenic Acids

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Harington and his colleagues (Clutton, Harington & Yuill, 1938, Butler, Harington & Yuill, 1940) started a series of studies on the immunological properties of certain protein complexes containing simple physiologically active substances as haptene groups. They showed that immunization with a thyroxine-protein complex produced resistance to the action of thyroxine. Similarly, immunization with an aspirin-protein complex produced resistance to the action of aspirin.

We have prepared the globulin complexes of (a) the 7-methyl ether of racemic bisdehydrodoisynolic acid (7-methoxy-2-methyl-1-ethyl-1,2,3,4-tetrahydrophenanthrene-2-carboxylic acid) and of (b) the 6-methyl ether of α , α -dimethyl- β -ethyl-allenolic acid (2-(6'-methoxy-2'-naphthyl)-1,1-dimethyl-*n*-valeric acid, Horeau & Jacques, 1947) with a view to determining whether immunization with the bisdehydrodoisynolic and the allenolic acid protein complexes will produce resistance to the bisdehydrodoisynolic and the allenolic acids themselves, and also to other uncombined oestrogens. Expressed schematically, it was desired to discover whether immunization with a globulin complex of oestrogenic acid *A* will confer resistance to the action of acid *A* as well as to oestrogenic acid *B* and other oestrogens. It was hoped that such a series of experiments would throw light on the part played by physiological properties and chemical constitution in the conferment of immunological properties on protein complexes.

The method we have used for coupling the oestrogenic acids with globulin has been that used by Butler *et al.* (1940) for coupling acetylsalicylic acid with globulin. The chlorides of both acids were treated at 0° in aqueous acetone solution with sodium azide to give the *azides*, which in both cases were very unstable to heat. These were then treated with horse serum globulin under alkaline conditions.

EXPERIMENTAL

7-Methyl ether of bisdehydrodoisynolic acid globulin complex

Chloride of 7-methyl ether of bisdehydrodoisynolic acid. The racemic 7-methyl ether of bisdehydrodoisynolic acid, m.p. 229–230°, was kindly supplied by Dr K. Miescher. The *Biochem.* 1949, 44

acid chloride was prepared according to Anner, Heer & Miescher (1946) using oxalyl chloride (Staudinger, 1908). After two crystallizations from benzene/light petroleum (b.p. 60–80°), colourless prisms (yield 80% of theory) were obtained, melting at 148–149° (Found: C, 71.9, H, 6.6, Cl, 11.1. Calc. for $C_{19}H_{21}O_2Cl$: C, 72.0, H, 6.7, Cl, 11.2%).

Azide. To a solution of 320 mg. of acid chloride in 18 ml. of acetone/80 mg. (1.25 mol.) of NaN_3 in 2.5 ml. of water were added and the clear reaction mixture was kept at 0° for 3 hr. The solution was then diluted with 50 ml. of ice water, when a very fine white precipitate formed. On extraction with cold ether, drying with $CaCl_2$ at 0° and evaporation to dryness in N_2 under reduced pressure without application of heat, 300 mg. (94% of theory) of colourless *azide* were obtained, melting sharply at 88° with evolution of N_2 . The *azide* is fairly stable at 0°, but loses N_2 on recrystallization from low-boiling solvents. Therefore, the freshly prepared crude product was dried in high vacuum for 1 hr. and analyzed (Found: C, 70.4, H, 6.6, N, 13.4. $C_{19}H_{21}O_2N_3$ requires: C, 70.5, H, 6.5, N, 13.0%).

Horse serum globulin solution. This was prepared by treating 600 ml. of fresh horse blood according to Reye (1911). The globulin solution (190 ml.) was stored in the ice chest with a thymol crystal (Found, by micro Kjeldahl: N, 1.00, 0.98%).

Coupling of the azide with horse serum globulin. To 16 ml. of the horse serum globulin solution 10 ml. of water were added. The mixture (0.62% N) was made alkaline to phenolphthalein with 2*N*-NaOH, 10 ml. of dioxan were added with stirring and the resulting solution was chilled in the ice chest. The *azide* (300 mg., 1 mmol.) was dissolved in 5 ml. dioxan and added slowly during 1 hr. to the globulin solution. A white precipitate formed. On addition of a further 16 ml. dioxan and 12 ml. water most of the precipitate redissolved. Throughout the experiment the reaction mixture was shaken mechanically and kept alkaline to phenolphthalein by dropwise addition of 0.1*N*-NaOH. At the end of the reaction (4 hr.) 9.7 ml. had been used (theory requires 10 ml.). The colloidal suspension was adjusted to pH 3.5 by addition of 2*N*-HCl when a white precipitate formed. After keeping at 0° for 1 hr. the mixture was centrifuged. The precipitate was suspended in 20 ml. of water and dialyzed for 3 days against running tap water. The complex was now divided into fractions soluble or insoluble at pH 9. The white sludge from the dialysis bag was made just alkaline to phenolphthalein with *N*-NaOH and shaken for 30 min. The suspension was centrifuged, the supernatant solution adjusted to pH 7.0 with *N*-HCl and stored in the ice chest with a thymol crystal. A 10 ml. portion of the cloudy solution (70 ml.) was evaporated to dryness by freeze drying under high vacuum over P_2O_5 . The white, solid residue (20 mg.) was ground finely and analyzed. No methoxyl was found. Thus the complex

required is not appreciably soluble at pH 9 and was present in the precipitate

The white precipitate from the last centrifugal treatment was dried in the same manner. The white residue contained 2.17% OCH_3 , corresponding to 19.6% 7-methyl ether of bisdehydrodisynolic residue. The ED_{50} in sprayed rats was about $5 \mu\text{g}$ using the standard technique employed at this Institute (Wilder Smith & Williams, 1947). An analysis of the original untreated horse serum globulin solution showed no methoxyl present.

*2-(6'-Methoxy-2'-naphthyl) 1,1-dimethyl
n-valeric acid globulin complex*

Chloride of 2-(6'-methoxy 2' naphthyl) 1,1-dimethyl n-valeric acid. The acid (1 g, colourless crystals, m.p. 138–139°, kindly supplied by Dr Horeau) was converted into the chloride by the method of Anner *et al.* (1946), the reaction was complete after 20 hr. After one recrystallization from light petroleum (b.p. 40–60°) 810 mg (76% of theory) of *acid chloride* were obtained in colourless needles, m.p. 63–64° (Found Cl, 11.7. $\text{C}_{18}\text{H}_{21}\text{O}_2\text{Cl}$ requires Cl, 11.6%).

Azide. The acid chloride (300 mg, 1 mmol) was treated with NaN_3 as described above. On evaporation to dryness in N_2 at 12 mm the *azide* was obtained as a colourless oil (300 mg) which solidified on cooling to -60° in solid CO_2 . No chlorine was detectable in the flame test and the substance decomposed with evolution of gas. For characterization and identification the corresponding symmetrical disubstituted urea was prepared as follows.

NN'-Di-[2-(6'-methoxy 2' naphthyl) 1,1-dimethyl butyl] urea. The acid chloride (300 mg, 1 mmol) was converted to the azide as described previously and the reaction mixture, containing the azide and NaCl in aqueous acetone, was refluxed on the steam bath. During 2 hr, 23 ml of N_2 (theory 25 ml.) were evolved, the evolution of gas then ceased. The acetone was evaporated off at normal pressure and ether was added. After washing with water, the ethereal layer was dried over Na_2SO_4 . On evaporation and

drying under high vacuum, 250 mg (93% of theory) of a residue were obtained, solidifying in colourless needles. After three crystallizations from light petroleum, b.p. 40–60°, 185 mg (69% of theory) of pure *substituted urea* were obtained, m.p. 72–73°. A sample was dried for 3 hr under high vacuum at 20° (Found N, 5.13. $\text{C}_{38}\text{H}_{44}\text{O}_3\text{N}_2$ requires N, 5.18%).

Coupling of the azide with horse serum globulin. Horse serum globulin solution (16 ml) was diluted with 10 ml water and made alkaline to phenolphthalein with 2N- NaOH . After adding 10 ml dioxan the solution was chilled in the ice chest. The azide (300 mg, 1 mmol.) was dissolved in 5 ml dioxan and added during 1 hr to the globulin solution with stirring, and the resulting suspension treated exactly as described for the coupling of bisdehydrodisynolic acid azide with globulin.

The fraction insoluble at pH 9 was freeze dried and analyzed (Found OCH_3 , 3.57%, corresponding to 31% acid residue). The ED_{50} was found to be about $15 \mu\text{g}$ in sprayed rats when tested by the technique used in this Institute (Wilder Smith & Williams, 1947).

SUMMARY

The coupling of the 7-methyl ether of racemic bisdehydrodisynolic acid and of 2-(6'-methoxy 2'-naphthyl)-1,1-dimethyl-n-valeric acid with horse serum globulin, with a view to the investigation of the immunological properties of the resultant complexes, has been described.

We are very grateful to the Council of the Middlesex Hospital Medical School for the provision of laboratory facilities for this work. One of us (W.H.) wishes to thank the Swiss Gnehm Stiftung for a scholarship held for a part of the period during which the research was carried out, and Prof. E. C. Dodds, F.R.S., who suggested this problem, for a grant.

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Seed Globulins of the Gramineae and Leguminosae

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1 SEED GLOBULINS OF THE MOST COMMON SPECIES OF THE GRAMINEAE AND THEIR DIFFERENTIATION IN THE SEED

The globulins in the seeds of different species of grasses do not occur in large amounts. The following values, taken from Osborne (1909), refer to seeds of wheat: glutenin, 4.68, gliadin, 3.96, globulin, 0.62, albumin, 0.39, 'proteose', 0.21%. It is evident that the globulins comprise only about 6% of the total protein. This naturally causes difficulties in carrying out investigations since yields are small.

A very valuable contribution to the knowledge of the vegetable proteins was made by Quensel (1942) in his investigations of the seed globulins of barley. His method for isolating them was based on Osborne's (1909) earlier work, and made use of the solubility of globulins in dilute salt solutions, and their precipitation by ammonium sulphate or by dialysis against water.

EXPERIMENTAL AND RESULTS

General method for the preparation of globulin fractions

Since Quensel's (1942) method of preparation was followed to a large extent in these investigations, only a brief account will be given here. Quensel extracted overnight at 4° C 800 g of barley meal with m NaCl , buffered with phosphate to pH 7.0. By fractional precipitation of the extract with solid $(\text{NH}_4)_2\text{SO}_4$, after clarifying by filtration, three fractions were obtained. By dissolving the precipitates in 0.2M NaCl , pH 7.0, and subsequent dialysis of the solution in a cellophan sac against distilled water, the globulins were separated from the albumins, low molecular and polydisperse material. In this way there could be obtained from barley seeds three fractions containing varying amounts of four well defined globulins called respectively α , β , γ and δ , in order of increasing molecular weight. These components were distributed amongst the different fractions in the following way: (1) 15%* saturation with $(\text{NH}_4)_2\text{SO}_4$, β rich fraction, (2) 40% saturation with $(\text{NH}_4)_2\text{SO}_4$, polydisperse fraction, (3) 70% saturation with $(\text{NH}_4)_2\text{SO}_4$, α , γ and δ rich fraction.

It may be noted here that the δ component, as shown by later investigations, only appeared occasionally in the sedimentation diagrams, and then only in small concentrations.

Method of investigation

This for the most part was based on ultracentrifugation (see Svedberg & Pedersen, 1940). In the presentation of sedimentation constants, Svedberg units have been used.

* 100% saturation = 760 g $(\text{NH}_4)_2\text{SO}_4/\text{l}$

($1 S = 10^{-13} \text{ cm sec}^{-1} \text{ dyne}^{-1}$) In the diagrams, the distance from the centre of rotation is plotted on the abscissa and the displacement of the scale divisions (Z) on the ordinate. To the left is the meniscus of the liquid at a distance of 5.8–6.0 cm from the centre of the rotor, and to the right is the bottom of the cell, about 7.2 cm from the centre. Since the displacement of the scale divisions is proportional to the concentration gradient in the cell, the area between the curve and the abscissa is proportional to the concentration. Each peak in the diagram corresponds to a protein component.

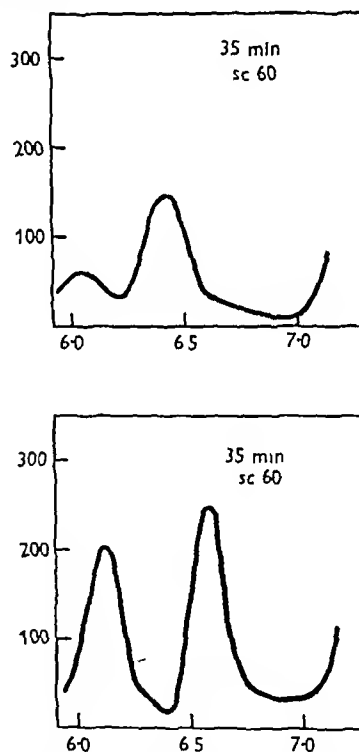


Fig 1 Sedimentation diagrams of barley globulins. Upper fraction obtained at 15% saturation with $(\text{NH}_4)_2\text{SO}_4$. Lower 70% saturation. In all the sedimentation diagrams in this paper the abscissae represent distance from the centre of rotation (cm), the ordinates represent the scale line displacement (μ), and sc = distance from scale (mm). Time of sedimentation is given in min.

Quensel (1942) found the following values of the sedimentation constants for the globulin components isolated from barley: $\alpha = 2.49 S$, $\beta = 6.21 S$, $\gamma = 8.30 S$, $\delta = 12.0 S$. Although he did not succeed in isolating any of these components separately, he worked out a method for determining the molecular weights of different components in

mixtures of this kind. The molecular weight is calculated from the Svedberg formula $M = \frac{RTs}{D(1 - V\rho)}$, where R = the gas constant, T = absolute temperature, V = partial specific volume, ρ = density of the solvent, M = molecular weight, s = sedimentation constant and D = diffusion constant. Taking $V = 0.72$ and the above values for s_{20} together with the values of the diffusion constants determined by Quensel's special method ($D_\alpha = 8.4$ and $D_\gamma = 4.4$ in 10^{-7} c.g.s. units), $M_\alpha = 26,000$ and $M_\gamma = 166,000$. The molecular weights for β and δ were estimated from their sedimentation constants to be $M_\beta = 100,000$ and $M_\delta = 300,000$. Typical sedimentation diagrams from barley are given in Fig. 1. The α and γ components always appear at the same time in the sedimentation diagrams, as mentioned above. Quensel tried to separate these two components, but this proved impossible with his methods, which included electrophoresis and centrifugation in an air centrifuge. He points out that no genetical relation between the components could be established, and further that there was no possibility of deciding on the basis of his investigations whether or not the different components were differentiated in the seed itself. The last question has been taken for investigation in this work.

The globulins of Gramineae other than barley

Since Quensel's work only applied to barley it is of interest to see in what degree these globulin components are found in other species of the same plant family. In investigations of this point the preparation scheme of Quensel has been followed, and the results which were obtained are given in Table 1 and

Table 1 *Globulins of species of Gramineae*

Species	Components				Sedimentation constants (S)			
	α	β	γ	δ	2.5	6.2	8.3	12.0
<i>Hordeum vulgare</i>	α	β	γ	δ	2.5	6.2	8.3	12.0
<i>Secale cereale</i>	α	—	γ	—	2.6	—	8.2	—
<i>Triticum vulgare</i>	α	—	γ	—	2.5	—	8.2	—
<i>Avena sativa</i>	α	—	γ	—	2.6	—	8.1	—
<i>Zea Mays</i>	α	—	γ	—	2.6	—	8.5	—
<i>Panicum miliaceum</i>	—	—	γ	—	—	—	8.5	—
<i>Phleum pratense</i>	—	—	γ	—	—	—	8.2	—
<i>Festuca pratensis</i>	—	—	γ	—	—	—	8.3	—
<i>Festuca rubra</i>	α	—	—	—	2.4	—	—	—

Fig. 2. The investigation had to be limited to the species occurring most commonly in commerce. It appears that the protein content varies greatly with the different species, and with some species is remarkably low. This is particularly true of *Phleum pratense* and the *Festuca* species, the seeds of which are very small, and seem to contain very small amounts of reserve protein. The preparation of globulins from these species is thus difficult and a negative result must not be considered as an absolute sign of their absence. Earlier work (Säverborn, Danielsson & Svedberg, 1944) has shown that malt contains the same components as barley, and the sedimentation diagrams from barley and malt are identical.

Quensel's investigations showed that the sedimentation constant of the γ component depends on concentration, i.e. $s = 8.30 - 0.6c$, where c is the percentage concentration and 8.30 is the sedimentation constant at infinite dilution ($c = 0$). The values in Table 1 have been corrected with the help of this equation. The concentrations have been obtained partly by measurement of the refractive index of the solution, and partly by calculation from the sedimentation diagram.

Hordeum vulgare (barley) is unique in this family, since it alone contains the β component, which occurs in high concentration. Barley is also the only species which contains the δ component, although only in low concentration.

The β component has some very interesting properties, which may possibly be connected with certain phenomena in the brewing of beer. The relations are not altogether clear, but it seems possible that the β component is part of the precipitate which is obtained on storing beer in the cold. Investigations at this Institute show that in this precipitate the β component may be combined with tannic acid. The β component is very stable towards many agents such as heat and enzymes, and it has been possible to show (Säverborn *et al.* 1944) that it survives the mashing process in the production of beer which causes degradation of the other proteins. The β component is distinguished from the other components by its content of 1.96% S, compared with 1.65% for the α and γ components. These proteins thus differ not only in molecular weight but also in chemical constitution. This partly explains the large differences on salting out with ammonium sulphate.

If these extra components in barley are disregarded, the different species within the family seem to have, to a large extent, the same seed globulins. It is to be noticed that the γ component is predominant in the species that have little reserve protein, for example, *Phleum pratense* and *Festuca pratensis*. This is understandable in the light of our experiments, when it was found to occur only in the embryo—a very small seed with little reserve protein would thus be expected to contain mainly the γ component.

Differentiation of the components in the seed

Experiments with wheat. The question of the differentiation of the globulin components defined by Quensel (1942) was partly solved by the work on wheat (Säverborn *et al.* 1944). The separation of the components was made possible by the fact that, in the milling of wheat, one can separate the embryo from the rest of the seed.

In the commercial milling of wheat a number of different fractions is obtained. Only the most interesting fractions have been investigated here. The

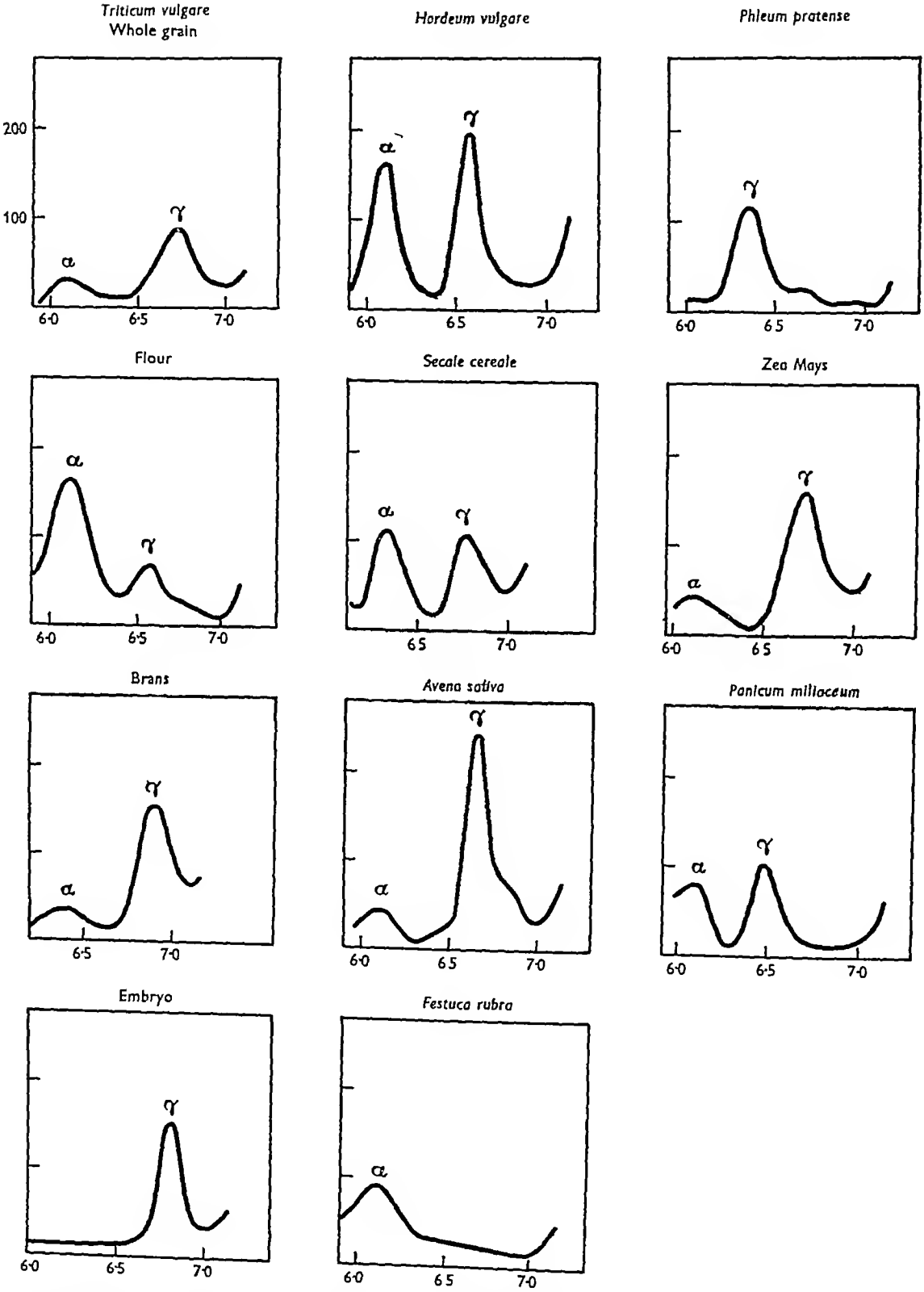


Fig 2 Sedimentation diagrams showing the composition of globulin solutions prepared by extraction of seeds from different species of the Gramineae. The four figures to the left refer to different fractions of seeds from wheat. Ordinates and abscissae as in top left.

results obtained earlier from investigations on the whole grain were compared with those obtained by the same method on the following fractions (a) bran fraction, (b) embryo fraction, (c) flour fraction with husk and embryo removed

The sedimentation diagrams of the globulins obtained from the various fractions were practically identical with those from barley, with the important exception that the β component was absent. Exactly the same sedimentation constants were obtained. Only in respect of the concentrations of the α and γ components could a difference be observed, as can be seen from Fig. 2. The γ component is obtained completely pure from the embryo fraction, while the flour fraction contains considerably more α than γ . Finally, the bran fraction contains γ in much higher concentrations than α . This fraction contains the so-called aleurone layer, which is particularly rich in proteins.

Experiments with barley In barley, the embryo cannot be separated directly by milling, but was obtained manually by excision with a razor blade. The two fractions (embryo and residual fraction) were investigated by the same method as before, using 100 g of embryo and 200 g of residual fraction for each experiment. The result obtained was in complete agreement with the above mentioned experiments with wheat seed. The sedimentation constants were naturally identical for both barley and wheat, and equal to those obtained by Quensel (1942) for solutions containing both components.

For barley, the α component occurs entirely in the residual fraction except for a low concentration in the embryo where it is present probably as a contaminant, being introduced along with the residual fraction which adheres to the embryo when the latter is removed from the seed manually. This finding is in accord with the observation that with wheat, as a result of the nearly perfect isolation of the embryo by the milling process, the α component is found only in the residual fraction.

The γ component occurs only in the embryo fraction of both wheat and barley, being isolated completely pure from wheat and only slightly contaminated with α , as mentioned above, in the barley preparation. Finally, the β component has been found only in barley seeds where it is present solely in the residual fraction.

The distribution of the different globulin components in the seeds of wheat and barley is shown in Table 2 and Fig. 3. It is of interest that the protein of highest molecular weight is in the embryo, the most vital part of the seed. It is perhaps premature to draw conclusions concerning the utilization of the proteins in the germination itself and in the formation of the young plant, but it is conceivable that the different components have different functions in germination. The latter causes a large diminution in

the concentrations of these components, and it is reasonable to suppose that they are directly broken down to smaller units, which can be easily utilized by the young plant.

Table 2 *Distribution of globulins within the wheat and barley seed*

	Embryo fraction			Residual fraction		
	α	β	γ	α	β	γ
Wheat	-	-	+++	+++	-	+
Barley	+	-	+++	+++	+++	-

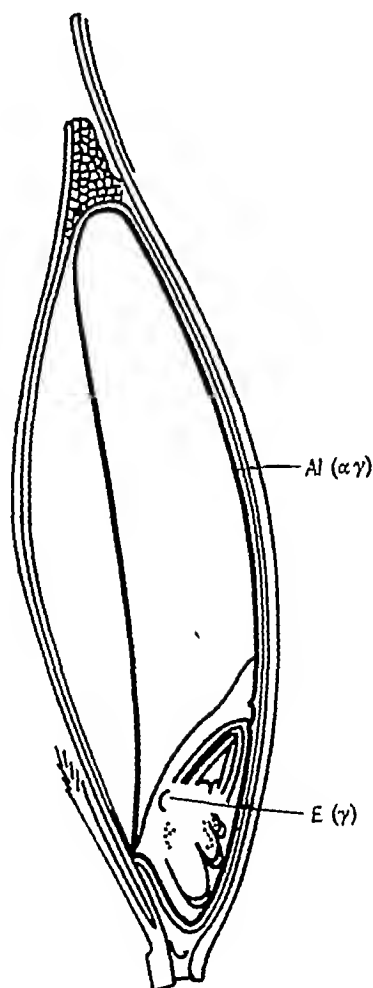


Fig. 3 Diagram showing the localization of the α and the γ components in seeds of wheat. Al, aleurone grains, E, embryo.

Whether there exists a biogenetical relationship between the globulin components described here is very difficult to decide. Many attempts have been made to degrade in different ways the purified γ component from wheat embryo in order to find out whether the α component can be obtained as an intermediate product, but without success. Ultrasonic vibration had no influence on the γ component, whilst among the proteolytic enzymes, papain

caused a very slow but continuous degradation under optimal conditions, and the process could easily be followed in the ultracentrifuge. No well-defined high-molecular intermediate products have been found under any circumstances, leaving the question of a possible relationship between the components unsettled.

(1937) Fig 4 shows the results of the diffusion measurements for the pure γ component from wheat embryo. The diffusion constant has been calculated by two methods, which give the values D_m and D_A . For a substance having ideal diffusion both methods must of course give the same value for D . The values in Table 3 have been calculated from the curves in

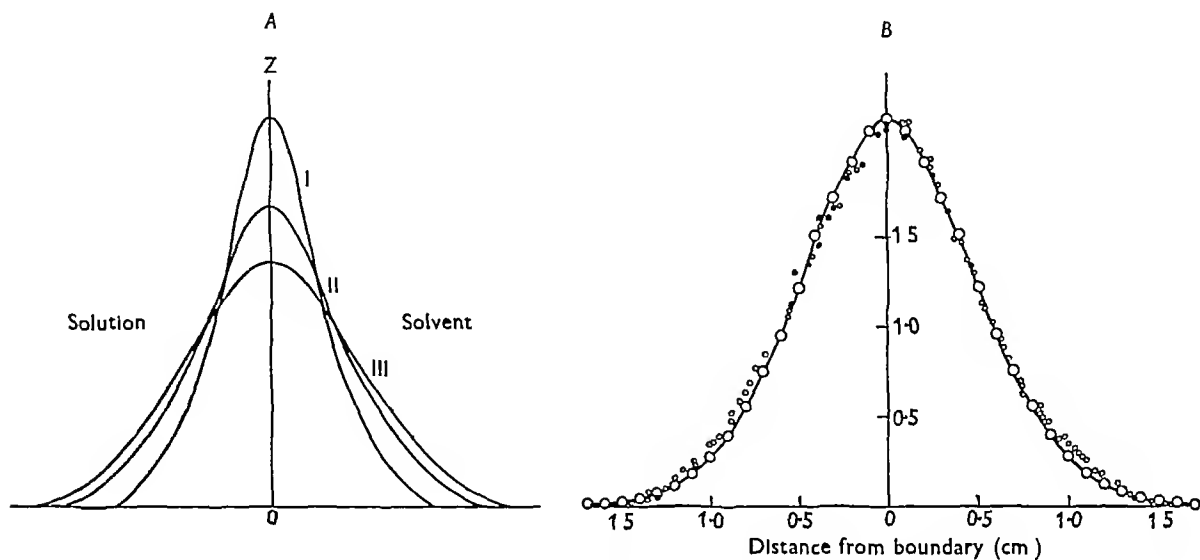


Fig 4 Diffusion diagrams of the γ component of wheat embryo. A, the curves marked I, II and III refer to exposures taken 29, 49 and 73 hr after the beginning of the diffusion process and show the concentration gradient as a function of the distance from the originally sharp boundary between solvent and solution (0). B, the diffusion curves I, II and III from A have been transformed to normal co ordinates, marked by small circles. The larger circles and the curve correspond to an ideal diffusion curve. Z=displacement of scale divisions

*Determinations of the molecular weights
of the α and γ components*

The methods described above for the isolation of the α and γ components have been used for the preparation of pure components for diffusion measurements. In this way α has been prepared from barley after removal of the embryo, while the γ component has been obtained from the wheat embryo.

In the determination of the molecular weight of the γ component, the concentration dependence of the sedimentation constant must be taken into consideration. In this investigation the value $s_{20}=8.7 S$ at infinite dilution was obtained for the γ component of wheat embryo, whereas Quensel (1942) found the value $8.30 S$, or about 5% less for γ from barley. However, in Quensel's experiments, the sedimentation of the γ component was disturbed by the presence of α , the contribution of which to the viscosity of the medium was not known and was accordingly omitted from the calculations. The sedimentation constant reported here for wheat γ is presumably correct since the preparation was pure. The apparent difference, therefore, in s_{20} for γ globulin from wheat and barley may be merely a result of experimental inaccuracy.

The diffusion measurements have been made with the help of the scale method worked out by Lamm

Fig 4. There is a steady decrease with time in the values for both D_m and D_A , which has persisted in later experiments. $D=3.6 \times 10^{-7}$ c.g.s. is taken as the average.

Table 3 D_{20} of the γ component of wheat embryo						
<i>(D is given in units of 10^{-7} c.g.s. at 20°)</i>						
Diffusion time (hr)						
	29	36	49	57	73	Average
D_m	3.72	3.71	3.52	3.41	3.44	3.56
D_A	3.76	3.71	3.57	3.58	3.46	3.62

Table 4 D_{20} of the α component of barley						
<i>(D is given in units of 10^{-7} c.g.s. at 20°)</i>						
Diffusion time (hr)						
	9	22	35	45	59	Average
D_m	7.99	7.64	7.64	6.81	7.08	7.43
D_A	7.78	7.35	7.34	6.79	7.10	7.27

From the values $s_{20}=8.7 S$, $D=3.6 \times 10^{-7}$ c.g.s., $V=0.72$ (Quensel, 1942) and $\rho=0.9982$, the molecular weight $M_\gamma=210,000$ is obtained. This exceeds

Quensel's value by 28 %, the difference being due to the fact that we used the isolated and completely pure γ component, which led to a greater accuracy of measurement. The values of D for the α component of barley are given in Table 4. The agreement

between D_m and D_A may be considered satisfactory. By using $s_{20} = 2.5 S$, $D = 7.4 \times 10^{-7}$ cgs, $V = 0.72$ (Quensel) and $\rho = 0.9982$ one gets $M_\alpha = 29,000$, a value which thus lies very near to that found by Quensel.

2 SEED GLOBULINS OF THE LEGUMINOSAE

The seeds of leguminous plants are very rich in proteins, containing 30–50 % total protein compared with 7–10 % for members of the Gramineae. It was assumed, therefore, that these globulins would be easier to prepare than those of the Gramineae, and this proved to be so.

Careful investigations of globulins from peas have been carried out by Osborne (1898). Apart from elementary analyses, Osborne studied chiefly the solubility and coagulation properties of the globulins. From these latter studies Osborne was able to isolate three globulin fractions from peas: (1) legumin, does not coagulate on heating to 100° , (2) vicilin, coagulates on heating to 95 – 100° , soluble in more dilute salt solutions than legumin, (3) legumelin, occupies an intermediate position with respect to globulins and albumins and is partly precipitated on dialysis.

In the experiments which will be described, Osborne's method of preparation has mainly been used, viz. precipitation with ammonium sulphate and dialysis. Our preparations, therefore, should be very similar to those which Osborne analyzed. The same scheme of preparation has been employed here as in the earlier experiments on the Gramineae; seeds of *Pisum sativum* have been mainly used.

Experimental and results

The sodium chloride extract of the ground seeds was at first precipitated with solid ammonium sulphate at three different degrees of saturation, 15, 40 and 70 %. Ultracentrifuge diagrams of the three fractions are all similar, and indicate two components (Fig. 5), with sedimentation constants about 7.5 and 12.5 S . Since the different fractions contained the same globulin components in about the same concentration the extract was subsequently precipitated directly at 70 % saturation. The following scheme of preparation has been used.

Finely ground seeds are extracted for 1 night at 4° with 10 times their weight of M NaCl, buffered with phosphate to pH 7. The solution is then centrifuged, and the extract filtered clear. Solid $(NH_4)_2SO_4$ is added with stirring to 70 % saturation (532 g. of salt/l.) and the precipitate centrifuged down. It is dissolved in 0.2 M NaCl, buffered to pH 7. After filtration, the solution obtained is dialyzed against running water in a cellophan sac. After 12–24 hr. the globulins have precipitated. They are recovered by centrifuging, the precipitate is redissolved, and the solution dialyzed once more until precipitation has occurred. The precipitate obtained from the second dialysis is dried *in*

vacuo over $CaSO_4$. In this way, a yellowish white powder is obtained which is very readily soluble in salt solutions at pH 7. A buffer with the following composition has been

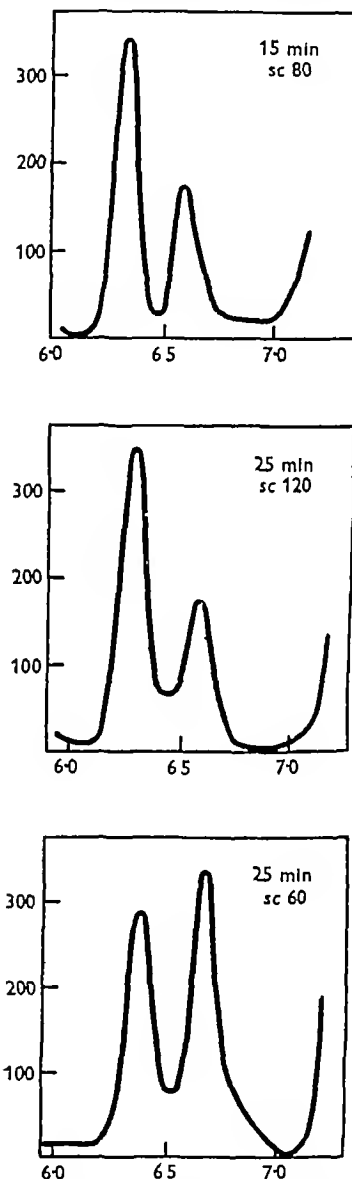


Fig. 5 Sedimentation diagrams showing the globulin composition in seeds of *Pisum sativum*: upper, 15 % saturation with $(NH_4)_2SO_4$; centre, 40 %; lower, 70 %.

used here: 0.2 M NaCl, 0.03 M Na_2HPO_4 , 0.02 M NaH_2PO_4 , pH 7.0. This buffer will be referred to as the standard buffer. The dried preparation can be stored indefinitely, and the solubility does not change with time (no change has been noticed during 1 year).

The yield varies of course from preparation to preparation, depending on how completely the substance precipitates during the two dialyses. Usually a yield of 1.5–2.0% of the pure components is obtained. Peas contain 21.5% total protein (Czapek, 1905). The globulins, therefore, comprise about 10% of the total protein.

mentation diagram of the 70% saturation fraction from peas.

In order to obtain more reliable values of the sedimentation constants, the centrifugations have been carried out at different concentrations. The results are given in Fig. 7. Thus, for the component with the higher sedimentation constant, no de-

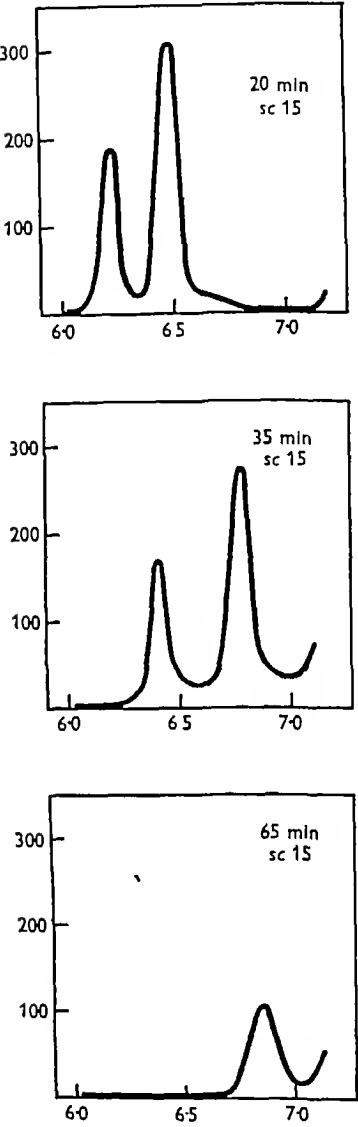


Fig. 6

Fig. 6. Sedimentation diagrams of the fraction obtained at 70% saturation with $(\text{NH}_4)_2\text{SO}_4$ (*Pisum sativum*). The diagrams correspond to exposures taken 20, 35 and 65 min. after the beginning of sedimentation at 65,000 r.p.m.

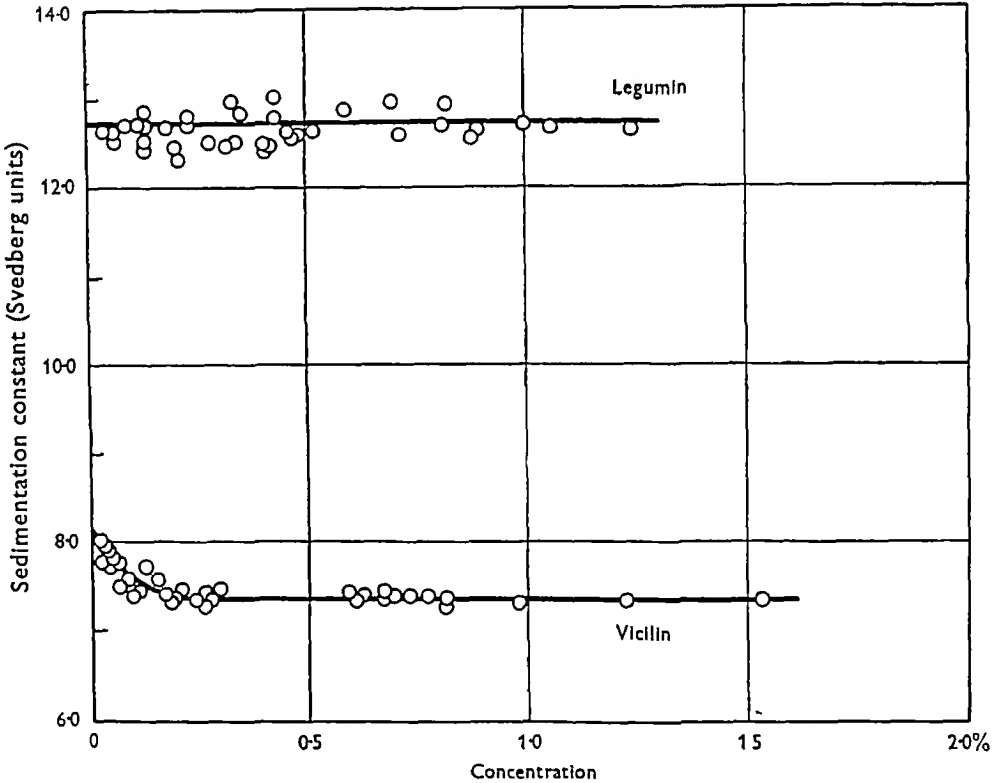


Fig. 7

Fig. 7. The relation between the sedimentation constants and the concentrations for the two globulin components in seeds from pea.

Investigation of pea globulins in the ultracentrifuge

As shown earlier (Fig. 5), two components are obtained on ultracentrifuging the globulin fractions prepared as above. Fig. 6 shows a typical sedi-

pendence of the sedimentation constant on concentration can be established, indicating that the particles are probably not highly asymmetrical. A value of the sedimentation constant for this component has been obtained by taking the average of the values given graphically in Fig. 7, the average of

forty values being $s_{20} = 12.64 S$. The sedimentation constant for the other component from peas shows, however, a distinct dependence on concentration. The sedimentation constant is usually given for infinite dilution, i.e. for zero concentration, and from Fig 7 the value $s_{20} = 8.10 S$ is obtained.

Attempts to separate the components

In the experiments described above both components have appeared at the same time in the diagrams. Experiments to isolate each component separately have been carried out in order that molecular weight determinations could be made.

buffer and precipitated with 11.0 g of $(\text{NH}_4)_2\text{SO}_4$. The precipitate was centrifuged off and dissolved in 10 ml of standard buffer, after which the solution was dialyzed for 2 days. The precipitate obtained was dissolved in 10 ml of standard buffer. The solution was designated *C* and investigated in the ultracentrifuge.

The results of these precipitation experiments are seen in Fig 8, which shows that by means of fractional precipitation it is possible to obtain the heavier globulin component relatively pure. The method is not, however, sufficiently effective. It has proved to be very difficult to remove the last traces of the lighter component, even if the method of separation described above is repeated.

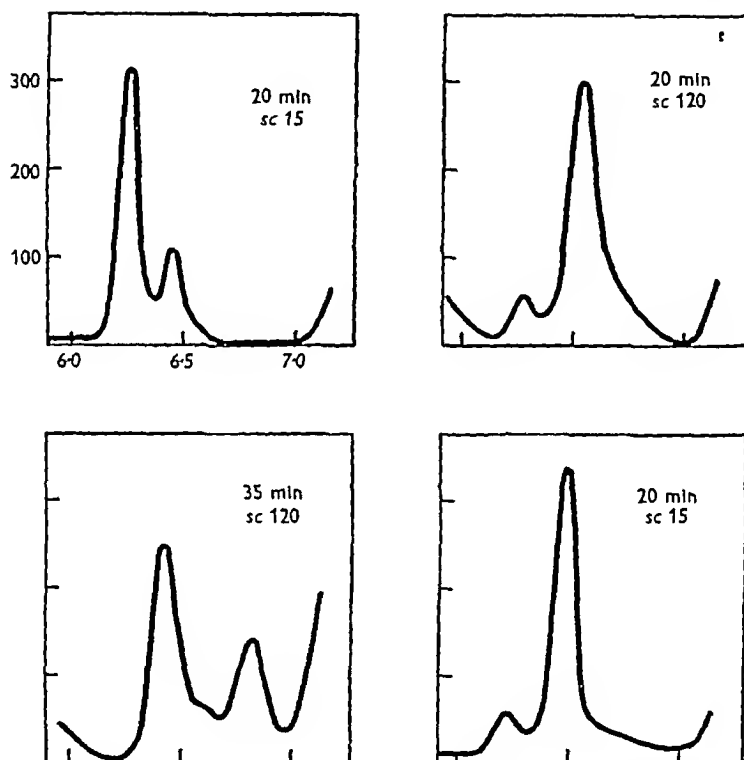


Fig 8 Sedimentation diagrams after fractional precipitation of vicilin and legumin with $(\text{NH}_4)_2\text{SO}_4$. Top left, initial solution, top right, solution A, bottom left, solution B, bottom right, solution C (see text). Ordinates and abscissae as in top left.

Fractional precipitation with ammonium sulphate The following experiment was carried out. 12.0 g of $(\text{NH}_4)_2\text{SO}_4$ were added to 25 ml of 0.6% pea globulin solution in the standard buffer, with stirring. The precipitate obtained was dissolved in 8.0 ml of standard buffer (solution A). To the centrifugate obtained above were added an additional 3 g of $(\text{NH}_4)_2\text{SO}_4$, after which the precipitate obtained was centrifuged down and dissolved in 10 ml of standard buffer (solution B). On further addition of salt no more precipitation was obtained.

Both the solutions, A and B were dialyzed against distilled water for 2 days at 4° . In this way the globulins precipitated quantitatively. After centrifuging down, the precipitates were dissolved in 10 ml of standard buffer, and investigated in the ultracentrifuge, whilst the supernatant fluid from A (9 ml) was diluted to 25 ml with standard

Separation by means of dilution with water at 0° The behaviour of solutions of pea globulin in standard buffer is strongly dependent on temperature. On cooling down to 0° a milk white suspension is obtained after about an hour, and if the solution is allowed to stand for a day or two at this temperature, a dense precipitate is obtained on the bottom of the vessel. Preliminary experiments showed that the substance precipitated in this way contained a relatively higher concentration of the heavier component than the original solution. A method of separation based on the above mentioned properties seemed possible. However, no satisfactory results were obtained merely by cooling the solutions, but instead the solutions were diluted with water at 0° . 10 ml of 1% pea globulin in standard buffer were cooled to 0° and diluted with distilled water at 0° . At a total volume of 35 ml (25 ml of water had been added)

a dense white precipitate was suddenly obtained. An additional 65 ml of water was added, after which the precipitate was centrifuged down at 0° in a refrigerator. Two fractions were obtained in this way. (A) The supernatant this was dialyzed against water in a cellophan sac at 4°. After 24 hr the precipitate obtained was dissolved in 10 ml of standard buffer and investigated in the ultracentrifuge. (B) The precipitate this was dissolved in 10 ml of standard buffer, and the precipitation repeated by the addition of 90 ml of water at 0°. This was repeated until the substance had been precipitated a total of five times. Finally, the precipitate was dissolved in 10 ml of standard buffer and investigated in the ultracentrifuge.

precipitates to a considerably greater extent than the lighter on dilution at a low temperature. The isolation of the two components is not, however, completely satisfactory if it is desired to prepare solutions for diffusion experiments, and other methods must be employed.

Separation by changing the pH Separation experiments described earlier have made possible the development of a new, effective method of separation. In a solution containing mainly the heavier component, prepared by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ (see Fig 8), the pH was slowly lowered. At pH 4.5-5 a precipitate was obtained

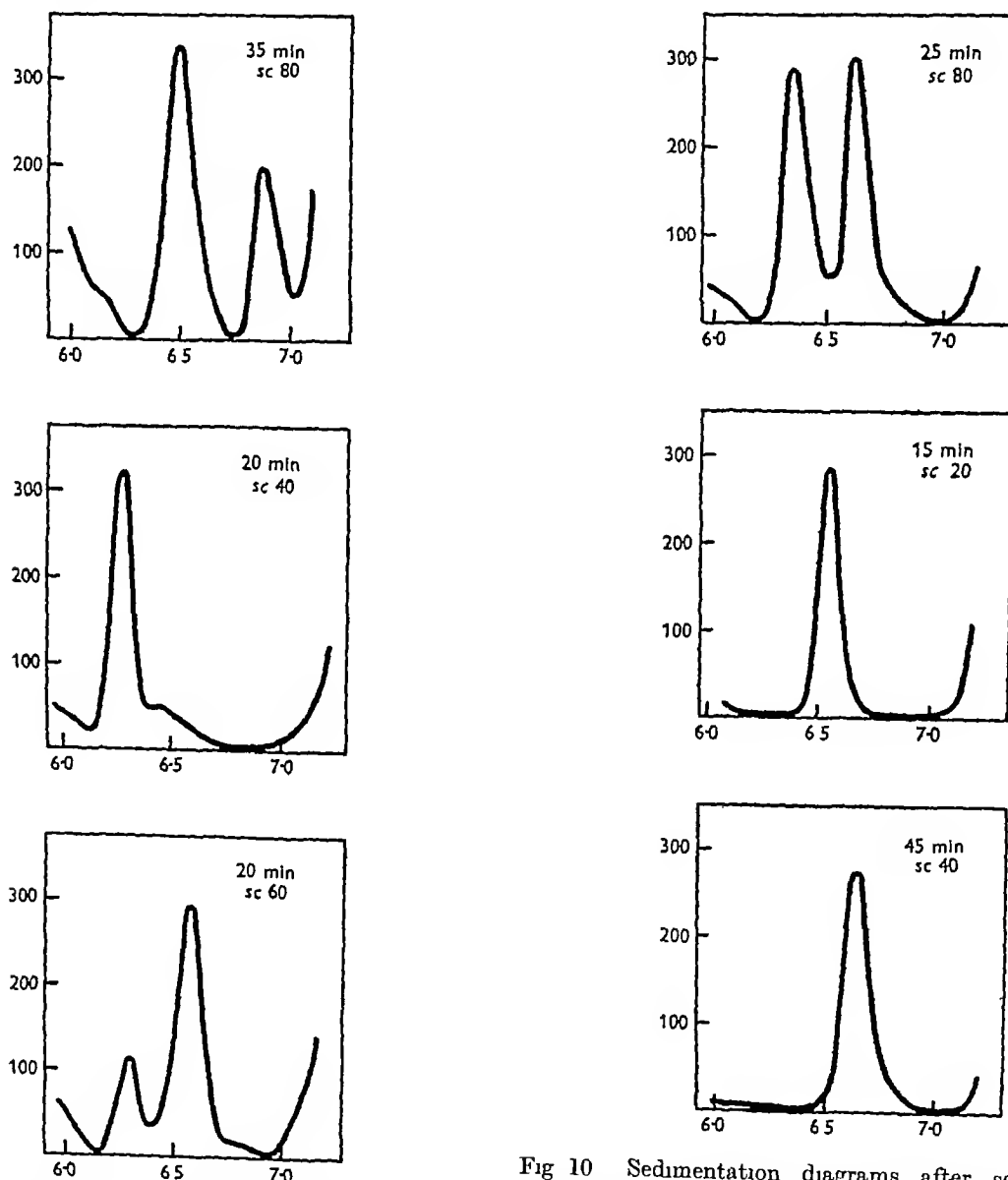


Fig 9 Sedimentation diagrams after separation of vicilin and legumin by means of dilution at 0°. Upper, initial solution, centre, supernatant, lower, precipitate (see text)

The results of the above precipitation experiments are shown in Fig 9. The heavier component pre-

Fig 10 Sedimentation diagrams after separation of vicilin and legumin by means of pH change. Upper, initial solution, centre, precipitate at pH 4.5 (legumin), lower, supernatant at 4.5 (vicilin) (see p 396 of text)

which was soluble on both sides of this precipitation range. In a solution containing the lighter component, prepared by dilution with water in the cold (see Fig 9), no precipitation occurs at any pH (only the range from pH 3 to 10 has been

investigated here in view of the risk of denaturation) This property has given rise to the most effective method so far for the separation of the two components A dried preparation, consisting of a mixture of approximately equal portions of the two components, is treated with a large excess of buffer of pH 4.5, with stirring Acetate buffers containing 0.2M NaCl have been used During this procedure the lighter component dissolves, while the other does not After 24 hr the heavier component is centrifuged off and dissolved in standard buffer This solution is dialyzed against water The centrifugate from the above centrifugation contains the lighter component, and this solution is also dialyzed After 1-2 days all the globulin has precipitated, and the two precipitates are dried in the cold On ultracentrifugation of the fractions so obtained the diagrams of Fig. 10 are obtained

This method of separation, based on isoelectric precipitation of the heavier component, is clearly the most effective of the three methods described here By repeated use of this method the two components have been completely separated from each other, which makes possible molecular weight determinations of both globulin components

Comparison with Osborne's globulin components

By isoelectric precipitation two solutions were prepared, one containing the lighter component and the other the heavier Coagulation experiments were carried out on these fractions, in order, if possible, to obtain some connexion between these two components and the globulin components defined by Osborne (1898) On boiling a solution containing the heavier component, no precipitate was obtained, i.e. the substance does not coagulate on warming to 100° This component should, therefore, consist of legumin, according to Osborne's definition On boiling, the component is of course destroyed, which is also shown by ultracentrifugation

On warming a solution containing the lighter component, precipitation is obtained at 95°, and, on boiling, all the protein precipitates The lighter globulin component consists, therefore, of vicilin In what follows the two components will be given these names Legumin is the component which has a sedimentation constant of 12.64 *S*, while vicilin has a sedimentation constant of 8.10 *S* at infinite dilution

As mentioned earlier, Osborne has pointed out that vicilin is soluble in more dilute salt solutions than legumin This is also clearly seen from the separation experiments by dilution with water at 0° It would seem to be proved by this that the globulin components isolated by Osborne, legumin and vicilin, are identical with the globulins from peas described in this paper Nothing corresponding to the legumelin defined by Osborne has, however, been detectable on ultracentrifugation

Determination of the molecular weight of vicilin

The initial solution for determinations of *D* and *V* of vicilin was prepared according to the method described earlier for the separation of vicilin from legumin by isoelectric precipitation of the legumin This method was applied several times so that the vicilin solution obtained was completely freed from legumin, as was established by ultracentrifugation

D was determined according to Lamm's (1937) scale method in a stainless steel cell designed by Claesson (1946), but somewhat modified The values of *D_m* and *D_A* obtained after different diffusion times are given in Table 5 The agreement between the two

Table 5 *Diffusion constant of vicilin*

(*D* is given in units of 10^{-7} c g s at 20°)

	Diffusion time (hr)				Average
	23	28	32	46	
<i>D_m</i>	4.44	4.18	4.22	4.30	4.29
<i>D_A</i>	4.31	4.20	4.18	4.17	4.22

different methods of calculation is good Taking an average value of $D = 4.26 \times 10^{-7}$ c g s, and an experimentally determined value of $V_{20} = 0.752$, the molecular weight of vicilin, using the value of the sedimentation constant found earlier, $s_{20} = 8.10$ *S*, and $\rho = 0.9982$, is found to be $M = 186,000$

Determination of the molecular weight of legumin

The initial solution for the determination of *D* and *V* in the case of legumin had been completely freed from vicilin by repeated isoelectric precipitation *D* was determined in the same cell and in exactly the same way as that of vicilin The results are given in Table 6 In this case also the agreement

Table 6 *Diffusion constant of legumin*

(*D* is given in units of 10^{-7} c g s at 20°)

	Diffusion time (hr)					Average
	23	25	28	45	48	
<i>D_m</i>	3.62	3.57	3.52	3.32	3.42	3.50
<i>D_A</i>	3.52	3.39	3.45	3.53	3.53	3.48

between the values of *D_m* and *D_A* is very good, indicating a high degree of monodispersity For the calculation of the molecular weight of the legumin the values used were $D = 3.49 \times 10^{-7}$ c g s and $s_{20} = 12.64$ *S* *V* was determined, as in the previous case, by Prof. C. Drucker, who obtained the value $V_{20} = 0.735$ With the value $\rho = 0.9982$, the molecular weight for legumin is found to be $M = 331,000$

Occurrence of vicilin and legumin in different species of the Leguminosae

The experiments described earlier to isolate seed globulins from different species of the Gramineae supported the working hypothesis that closely related plants contain the same types of protein. A further confirmation of this hypothesis seems to be given by the following investigations on different species of the Leguminosae.

Experiments to isolate the globulins from seeds of different Leguminosae have been carried out in exactly the same way for all the species. Owing to the difficulties in obtaining a sufficient amount of seed of some of the species, the amount of initial substance has varied between 3 and 10 g. The proportion of amount of extraction buffer to amount of seed has been kept constant throughout. The scheme of preparation has been the same as in the experiments described earlier for the isolation of vicilin and legumin from peas, *Pisum sativum*, whereby a solution containing both components is obtained.

Table 7 *Sedimentation constants of the globulin components of various Leguminosae*

(Results in Svedberg units)

<i>Acacia alata</i>	1 25	7 90	11 63	—
<i>A. decipiens</i>	—	8 02	12 70	—
<i>A. Farnesiana</i>	—	8 04	—	—
<i>A. longifolia</i>	2 72	7 59	—	18 04
<i>A. penninervis</i>	1 46	7 39	—	18 75
<i>A. saligna</i>	—	7 76	13 67	—
<i>A. verticillata</i>	—	7 77	—	—
<i>Arachis hypogaea</i>	1 93	8 40	13 05	—
<i>Astragalus galegiformis</i>	—	8 33	13 17	—
<i>Cytisus Laburnum</i>	—	8 08	14 02	—
<i>C. supinus</i>	1 84	8 03	13 38	—
<i>Dolichos Lablab</i>	—	7 33	11 66	—
<i>Ervum Lens</i>	—	7 25	13 18	—
<i>Genista tinctoria</i>	—	8 54	13 34	20 31
<i>Glycine Soja</i>	—	7 87	13 06	—
<i>Lathyrus Clymenum</i>	—	7 55	13 00	—
<i>L. odoratus</i>	—	7 64	12 00	—
<i>L. sativus</i>	—	7 46	13 04	—
<i>L. silvestris</i>	—	7 48	12 97	—
<i>Lotus Tetragonolobus</i>	—	8 32	13 07	—
<i>Lupinus albus</i>	—	8 24	12 29	—
<i>L. angustifolius</i>	—	8 20	13 05	—
<i>L. luteus</i>	—	8 30	11 53	—
<i>L. polyphyllus</i>	—	8 69	12 20	—
<i>Medicago sativa</i>	—	6 77	11 41	—
<i>Phaseolus coccineus</i>	4 29	7 39	12 16	—
<i>P. nanus</i>	—	6 55	10 10	—
<i>P. vulgaris</i>	4 87	7 26	11 02	—
<i>Pisum sativum</i>	—	8 10	12 64	—
<i>Trifolium hybridum</i>	—	7 66	12 90	—
<i>T. pratense</i>	—	7 69	11 22	—
<i>T. repens</i>	—	7 27	—	18 20
<i>Vicia Faba</i>	—	7 12	11 80	—
<i>V. sativa</i>	—	7 09	11 91	—

As follows from Table 7 and Fig 11, vicilin and legumin occur in the seeds of practically all the species investigated of the Leguminosae. The most

important exceptions are a few *Acacia* species (*A. longifolia*, *A. penninervis*, and *A. verticillata*). In these species, legumin is absent, while in the other four *Acacia* species it has been possible to isolate legumin, even although in rather small quantities. On comparing *Acaciae* with the other species of the Leguminosae it is seen that the former hold a unique position in this family, they are, in fact, sometimes considered as a subfamily within the Leguminosae, and this may be justified to a certain extent by the protein composition of the seeds. Furthermore, it has not been possible to isolate legumin from the seeds of *Trifolium repens*. However, this apparent absence of legumin may simply result from difficulties in preparation, since the various *Trifolium* species are relatively poor in proteins.

It is seen from Table 7 that in some cases globulin components have been isolated with a sedimentation constant of $s_{20} \approx 2 S$. These components probably constitute a degradation product of legumin. According to investigations, as yet unpublished, a component of globulin nature with a sedimentation constant of $1.9 S$ is obtained by dissolving legumin in a buffer of pH 3. The same component is obtained from legumin by protracted heating at 100° . In some cases, a component of $s_{20} \approx 18 S$ has also been obtained, though always in very low concentration, it is found in *Pisum sativum*, but it has not been possible to separate it from vicilin and legumin. Whether it exists in the seeds, or results from complex formation between vicilin or legumin, is not yet clear. Experiments to answer this question seem, however, to indicate the latter possibility.

It is also interesting to compare the relative concentrations of vicilin and legumin in the different species. Closely related species have approximately the same relative concentrations of the two components. In the various *Lathyrus* species, for example, legumin always predominates, as is also the case for the two *Vicia* species investigated. These two genera are, of course, botanically very near to each other. In the various *Lupinus* species it is the vicilin which is in greater concentration, and this also applies to the *Phaseolus* and *Trifolium* species.

If one specially considers *Vicia sativa* it is seen from the sedimentation diagram that vicilin is found in somewhat lower concentration than legumin. This is contrary to the conception of Osborne & Campbell (1898), according to whom vicilin should be completely absent from this species.

In order to obtain more accurate values of the relative concentrations of vicilin and legumin, a new method is being developed for determining the concentrations of these components in solutions containing both, based on the fact that legumin contains about five times more tryptophan than vicilin. By measuring the absorption in the ultraviolet, the

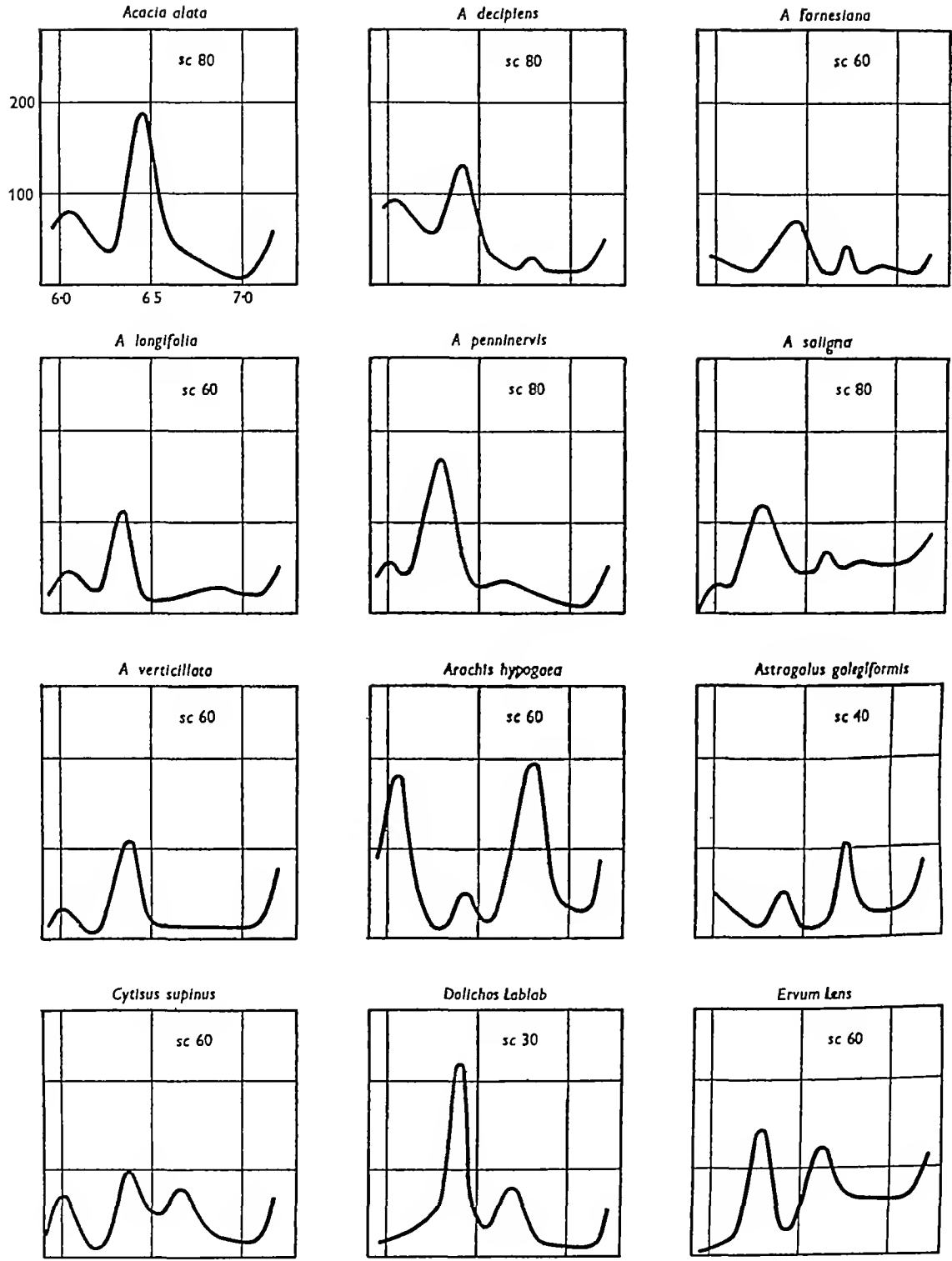


Fig 11a

Fig 11a-c Sedimentation diagrams showing the composition of globulin solutions prepared by extraction of seeds from different species of the Leguminosae Ordinates and abscissae as in top left

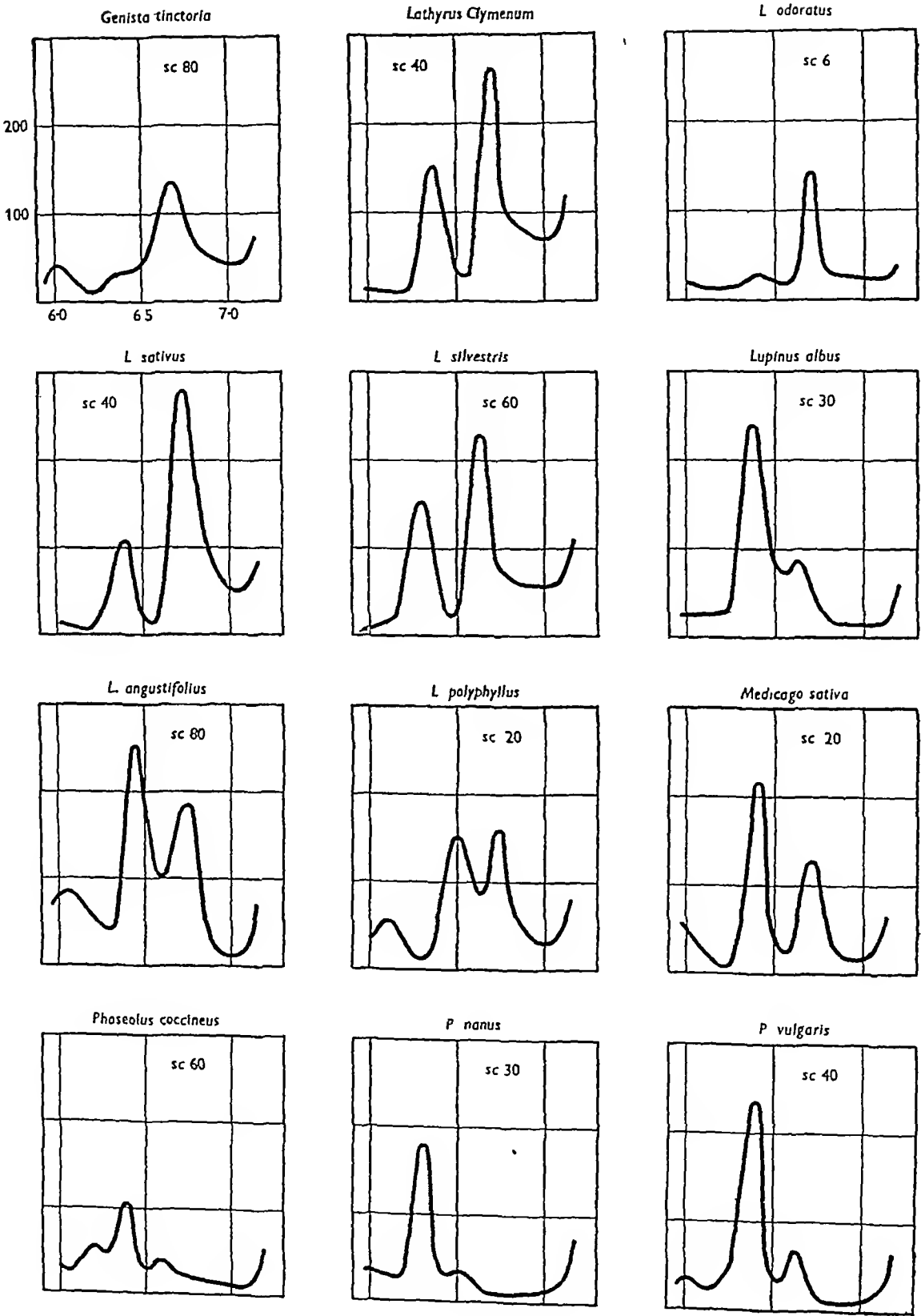


Fig 11 b For legend see p 398

relative concentrations of vicilin and legumin can be determined. The results will be published in a later paper.

components have been isolated. Different methods have been developed for the separation of these components, which are identical with vicilin and

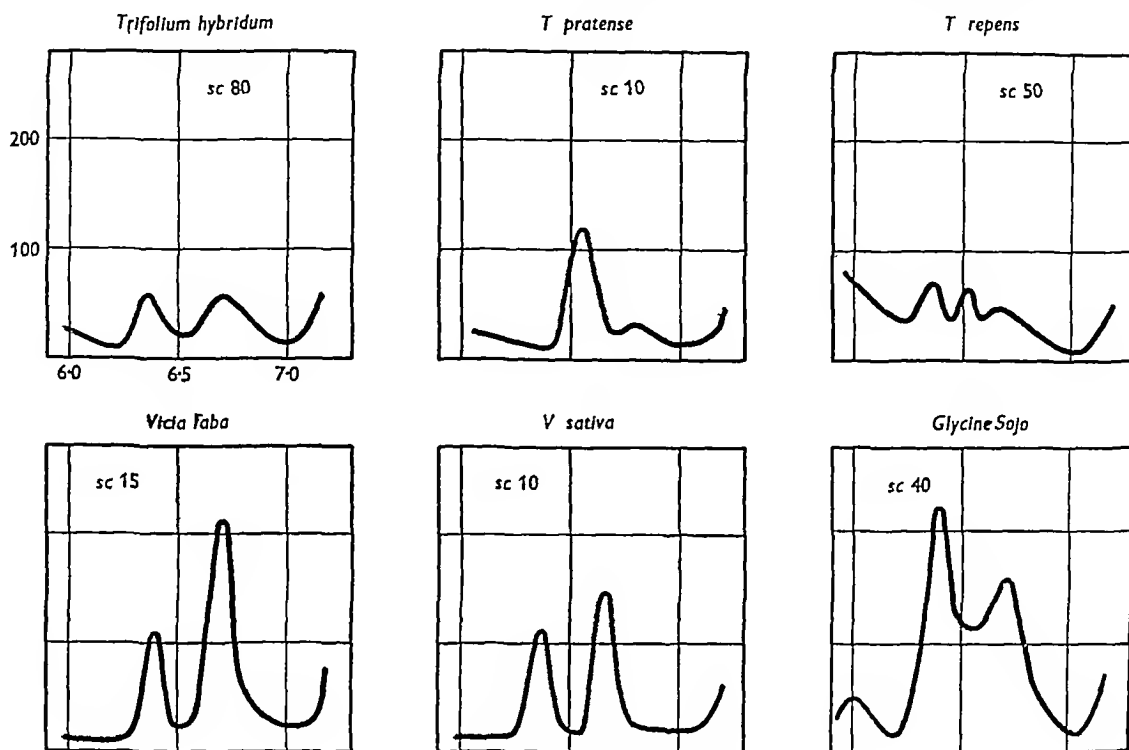


Fig. 11c For legend see p. 398

SUMMARY

1 The seed globulins of nine different species in the family Gramineae have been investigated. Two well defined globulin components, α and γ , were isolated. The α component was found in six and the γ component in eight species. Seeds of barley contain two other components, β and δ , which were not found in any other species of this family.

2 These components occur in different parts of the seed itself. Investigations on different fractions from seeds of wheat and barley have shown that the γ component alone occurs in the embryo fraction and the α component in the residual fraction. The husk fraction contains γ in higher concentration than α .

3 Determinations of the molecular weight of the purified components have given the values $M = 29,000$ for α and $M = 210,000$ for γ .

4 From the pea, *Pisum sativum*, two globulin

legumin, first defined and investigated by Osborne (1898, 1909).

5 The molecular weight of vicilin was found to be 186,000, and that of legumin 331,000.

6 The seed globulins from thirty four different species in the family Leguminosae have been investigated. Vicilin and legumin were found in all, except in some *Acacia* species and in *Trifolium repens*. In a few cases degradation products and complex compounds of these components have been isolated.

7 These investigations seem to confirm the hypothesis that closely related plants contain the same seed globulins.

The author wishes to thank Prof. Svedberg for the great and stimulating interest which he has shown in this work, and for the privilege of working in his laboratory. The expenses connected with these investigations were defrayed by the AB Stockholms Bryggerier and the Rockefeller Foundation.

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Carotenoids of Loquat (*Eriobotrya japonica* Lindl.)

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The pigments of loquat (*Eriobotrya japonica*) do not appear to have been investigated heretofore. Loquat is a valued subtropical fruit and is grown abundantly in Northern India either from seed or from grafts. It is yellow or orange, 3–7 cm long with a thin skin and having one to five (commonly three) seeds separated by membranous dissepiments. The fruit is pear shaped and ripens towards the end of the cold season.

After saponification of the fruit pulp, a colorimetric analysis of the total extract gave gross carotenoid values which varied from 5.1 to 56.5 $\mu\text{g/g}$. After a chromatographic separation, the true β -carotene content was found to be only 36–60% of the total pigments. From the carotene fraction, three other pigments besides β carotene were isolated, one of which was cryptoxanthin and the other two were stereoisomers of β -carotene, viz. neo β -carotene U (adsorbed just above the β carotene zone) and neo- β -carotene B (adsorbed below the β -carotene zone). The hypophasic pigments have all been classed as xanthophylls other than cryptoxanthin.

EXPERIMENTAL

An accurately weighed amount of the pulp of the fresh fruit (5–6 g) was thoroughly ground with chemically pure white sand under methanol. The ground material was quantitatively transferred to a digestion flask, and saponified in the cold with 100–125 ml of concentrated methanolic KOH with continuous agitation to facilitate thorough digestion. The saponified mixture was filtered, and the residue extracted alternately with light petroleum (b.p. 40–60°) and methanol till the washings were colourless. Distilled water (100 ml) was then added to the combined light petroleum

and methanol fractions in a separating funnel and shaken gently. After complete separation of the two layers the methanolic solution was drawn off and treated with a fresh portion of light petroleum in a separating funnel. This process was continued until all the carotenoid pigments were transferred to light petroleum. The combined light petroleum fractions were washed free from soaps and alkali with distilled water. The first washings were re-extracted with light petroleum and returned to the principal extract. Xanthophylls were separated by repeated extractions with 90% (v/v) aqueous methanol. The methanol fraction was distilled under reduced pressure in an atmosphere of N_2 . The residual pigment mixture was taken up in light petroleum and the amount present was determined colorimetrically. The epiphasic pigments were washed with distilled water to remove the last traces of methanol and dried over anhydrous Na_2SO_4 . The extract was concentrated to 8–10 ml under reduced pressure in the presence of N_2 . A few crystals of quinol were added before concentration to prevent oxidation. The solution containing the pigments was passed through an adsorption column of activated alumina (obtained from Scientific Supplies (Bengal) Co., Ltd.). The chromatogram was developed by a light petroleum-benzene mixture (1/1, v/v). The alumina column was cut to separate different coloured zones, and each portion was eluted with petroleum ether containing 2–3% (v/v) ethanol, and made up to volume. The pigments in the eluates were determined colorimetrically using 0.04% $\text{K}_2\text{Cr}_2\text{O}_7$ as standard.

For the study of absorption spectra, the separated pigments were purified by two chromatographic adsorptions. The absorption spectra of the purified pigments were studied in light petroleum using a Hilger Nutting visual spectrophotometer. The pigments constituting the different zones were as follows:

Zone I This was wide and orange yellow in colour. The pigment was extracted and shown to be identical with cryptoxanthin (maxima in light petroleum, 483 and

Table 1 Carotenoids of loquat (*Eriobotrya japonica* Lindl.)
(The figures denoting the pigment content refer to $\mu\text{g/g}$ of the fresh fruit pulp.)

No	Variety	Sum of individual pigments ($\mu\text{g/g}$)	Xanthophylls other than cryptoxanthin		Cryptoxanthin		Neo β carotene U		β carotene		Neo β carotene B	
			($\mu\text{g/g}$)	(% of total)	($\mu\text{g/g}$)	(% of total)	($\mu\text{g/g}$)	(% of total)	($\mu\text{g/g}$)	(% of total)	($\mu\text{g/g}$)	(% of total)
1	Pale yellow	5.1	1.4	27.4	1.1	21.5	—	—	2.1	41.2	0.5	9.8
2	Yellow	5.8	1.8	31.0	1.0	17.2	0.3	5.2	2.3	39.6	0.4	6.9
3	Orange yellow	10.5	3.2	30.5	2.4	22.8	—	—	4.1	39.0	0.8	7.6
4	Pale orange	35.7	3.9	10.9	5.6	15.7	1.8	5.0	20.8	58.2	3.6	10.1
5	Orange	37.0	6.6	17.8	12.5	33.8	0.9	2.4	13.5	36.5	3.5	9.4
6	Deep orange	56.5	4.7	8.3	11.6	20.5	1.6	2.8	33.7	59.6	4.9	8.7

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450 $m\mu$) since it yielded a single band on mixed chromatography with cryptoxanthin obtained from yellow maize (Sadana, 1946)

Zone II This was an orange coloured band. The pigment was identified as neo β carotene U (maxima in light petroleum 480 and 450 $m\mu$, Sadana, 1949)

Zone III This was reddish orange in colour. The pigment was extracted and identified as β carotene by its absorption spectrum (maxima in light petroleum 482 and 452 $m\mu$)

Zone IV This was a brownish yellow band. The pigment was extracted and identified as neo β carotene B (maxima in petroleum 470 and 443 $m\mu$). It yielded a single band on mixed chromatography with neo β carotene B prepared by HCl treatment of β carotene (Sadana, 1949)

Results of analyses of six varieties of loquats varying in colour from pale yellow to deep orange are shown in Table 1

SUMMARY

Pigments separated chromatographically and identified from different varieties of loquats include, besides 'xanthophylls other than cryptoxanthin', cryptoxanthin, β carotene, neo β -carotene U and neo- β -carotene B. Among the pigments present, cryptoxanthin and β carotene are the principal ones forming 15.7–33.8 and 36.5–59.6% of the total pigments.

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Improved Separation of Sugars on the Paper Partition Chromatogram

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Our object has been to develop methods for the quantitative analysis of the mixtures of sugars present in the polysaccharides of the cell wall of fruits, these mixtures frequently include glucose, galactose, xylose, mannose and arabinose, as well as galacturonic and glucuronic acids. The work of Partridge (1946, 1948) has demonstrated the effectiveness of the paper chromatogram as a means of separating reducing sugars, whilst the work of Hirst, Jones & Flood (1947) and Hawthorne (1947) has shown that the amounts of sugars can be estimated within $\pm 5\%$ once they have been separated on the paper chromatogram.

It was found in preliminary experiments that none of the solvents previously used in this work was in fact capable of separating all the sugars known to be present in the mixtures under investigation. A large number of solvents was then investigated, and this study led not merely to the development of two new solvents having the required specificity, but also to a more selective method of operating the paper partition chromatogram. The new method involves the use of solvents in which sugars give R_F values of about 0.2. Under such conditions the selectivity defined in terms of the ratio of the R_F values for the pair of sugars to be separated is much improved. Separation of sugars with such low R_F values would need exceedingly long papers if run in the normal manner, but by allowing the solvent to drip off the end of the paper

the difficulty has been avoided. Solvents are chosen which have a low viscosity so that the solvent flows quickly through the paper.

To obtain solvents which give R_F values about 0.2 for any particular pair of sugars, it is convenient to use three component systems so that the partition coefficient, and with it the R_F value, can be adjusted to the desired value. Such solvents have been used previously by Martin & Synge (1941), Isherwood (1946) and Partridge (1946, 1948). Usually two of the components are immiscible whereas the third is miscible with either of the other two. By varying the amount of the third component it is possible to adjust the partition coefficient over a wide range. The two new solvents, ethyl acetate-pyridine-water and ethyl acetate-acetic acid-water are mixtures of three components and in addition have a low viscosity.

EXPERIMENTAL

Apparatus The apparatus was similar to that described by Partridge (1948). The main difference was that a small hole was bored through the lid of the chamber immediately above one end of the stainless steel trough so that a pipette could be inserted to deliver solvent to the trough. The hole was normally closed with a tight fitting bung. Whatman no. 1 filter paper was used for the strip chromatograms, one end being cut to a point to facilitate even drainage when the solvent front had reached the bottom and was dripping from the paper. The sugar solutions used were roughly 1% (w/v) with respect to each individual sugar and were

applied to the paper with a micropipette (3–7 μ l) so that the size of the spots could be standardized. The whole apparatus was kept in a constant-temperature room at $20^\circ \pm 0.25^\circ$, as it was found that changes in temperature altered the R_F values for the sugars in an irregular manner, especially if the critical temperature of the mixture of solvent and water was close to that of the room.

Reagents The phenol and *s* collidine were purified as described by Partridge (1946, 1948). The *sec*-butanol was purified by distillation through a vacuum-jacketed fractionating column, the middle third of the distillate being used for the paper chromatograms. This fraction had a constant boiling point. *n*-Butyric acid was treated similarly. The critical temperature of a mixture of the purified *n*-butyric acid and water was found to be 24.3° . The accepted figure is 24.5° .

Solvents which contained only one component besides water were equilibrated against water at 20° . In the case of solvents containing three components, definite volumes of each component were mixed and when equilibrium between the two phases that resulted had been attained, the non-aqueous phase was separated and used as solvent without further treatment.

Procedure The spots of sugar solution were added to the paper from a micropipette (3–7 μ l) in a similar manner to that described by Hawthorne (1947). The papers were allowed to dry, and were then hung from the trough in the chamber, no solvent being present. Sufficient of the non-aqueous and aqueous phases was now added to the bottom of the chamber to ensure that the papers would reach equilibrium with the solvent. The lid was placed in position and the apparatus left overnight. After this preliminary equilibration the bung was removed from the small hole in the lid, and solvent added to the trough from a pipette, care being taken to avoid loss of the vapour from inside the chamber. The bung was then replaced. The subsequent treatment of the papers follows closely that described by Partridge (1948).

RESULTS

Variation of R_F value with solvent

Results for rhamnose, xylose and glucose are recorded in Table 1. These sugars were chosen because they were conveniently spaced within the range of R_F values obtained for the simple sugars. The solvent front was not allowed to run off the paper.

These results showed that the absolute R_F values were directly dependent upon the molar fraction of water in the non-aqueous phase. They also showed that, as the molar fraction of water in the non-aqueous phase decreased, the ratio of the R_F values for the pairs of sugars approached a definite maximum. For practical purposes this maximum was reached when the R_F values were less than 0.2, which meant that the possibility of separating two similar sugars increased as the absolute R_F values were reduced to 0.2. A paper chromatogram operated under this restriction did not give a sufficient separation of the sugars if the solvent front remained on the paper because the distance between the centres of the spots was often not more than 1–2 cm. However, if the solvent front was allowed to run off the paper so that the spots moved 10–20 cm, then the distance between the centres increased to 5–10 cm.

Separation of the sugars

From the preceding paragraph it appears that the best conditions for the separation of the sugars are when the R_F values are less than 0.2 and the solvent front is allowed to run off the end of the paper so that the spots move 10–20 cm. The main drawback is that the time required for the spots to move apart on the paper can be considerable if the solvents used have a high viscosity, e.g. cyclohexanol. It is essential, therefore, to use solvents which have a low viscosity in order that the solvent front may move as quickly as possible. A comparison of the viscosity of various solvents with the rate of movement of the solvent front is given in Table 2. The viscosities given are those for pure liquids while the velocity of the solvent front is for the same liquid saturated with water. The influence of viscosity on the separation of the sugars has been calculated by combining the figures for the velocity of the solvent front with the R_F values given in Table 1, the assumption being made that the rate of flow of solvent through the paper and the R_F value remain constant.

Table 1 Variation of R_F values of sugar with solvent at 20°

Solvent	Water in non aqueous phase Mol fraction	R_F value			R_F Rhamnose R_F Xylose	R_F Xylose R_F Glucose
		Rhamnose	Xylose	Glucose		
Ethyl acetate	0.132	0.01	0.00	0.00	—	—
Methyl acetate	0.269	0.075	0.04	0.00	1.9	—
Methyl ethyl ketone	0.305	0.095	0.05	0.0025	1.9	2.0
Benzyl alcohol	0.387	0.11	0.06	0.03	1.8	2.0
cycloHexanol	0.432	0.15	0.06	0.03	2.5	2.0
<i>m</i> Cresol	0.471	0.27	0.15	0.07	1.75	2.1
<i>n</i> Butanol	0.508	0.22	0.125	0.07	1.7	1.8
<i>tert</i> -Amyl alcohol	0.609	0.32	0.245	0.145	1.30	1.7
Phenol	0.668	0.59	0.44	0.39	1.34	1.13
Quinolone	0.680	0.41	0.31	0.19	1.32	1.6
<i>sec</i> Butanol	0.707	0.41	0.31	0.26	1.32	1.27
<i>n</i> -Butyric acid	0.778	0.51	0.44	0.37	1.16	1.19
<i>s</i> Collidine	0.846	0.59	0.50	0.44	1.18	1.14

Table 2 Calculated distance the centre of a sugar spot should move in 24 hr for different solvents at 20°

Solvent	η (Centipoises)	Velocity of solvent front (cm/hr)	Distance (cm)			
			Solvent front	Rhamnose	Xylose	Glucose
Methyl acetate	0.38	12	288	21.6	11.5	0.0
Ethyl acetate	0.45	10	240	2.4	0.0	0.0
Methyl ethyl ketone	0.43	10.8	258	25.5	12.9	6.4
Water	1.0	3.75	—	—	—	—
isoButyric acid	1.3	2.25	54	27.5	23.7	19.9
n Butanol	3.0	2.6	62	13.6	7.8	4.4
sec Butanol	3.7	2.6	62	25.6	19.2	16.2
tert Amyl alcohol	4.7	1.25	30	9.6	7.3	4.3
Benzyl alcohol	5.8	1.6	38.4	4.2	2.3	1.1
Phenol	11.7	1.5	36	21.2	15.8	14.1
m Cresol	20.0	1.1	26.5	7.1	4.0	1.8
cycloHexanol	68.0	0.65	15.6	2.3	0.9	0.4

Table 3 Separation of sugars using three component solvents

Mixture prepared (in vols) Non aqueous phase used as solvent	Duration of test (hr)	Movement of the centre of the sugar spot from the starting point (cm)							
		Rhamnose	Xylose	Arabinose	Mannose	Fructose	Glucose	Galactose	Galact- uronic acid
tert Amyl alcohol 3	20	8.6	7.0	5.4	5.9	5.9	4.6	4.1	3.2
Water 3									
Acetic acid 1									
Benzyl alcohol 3	22	12.8	7.8	7.5	5.7	7.2	4.7	4.6	2.6
Water 3									
Acetic acid 1									
Chloroform 4	24	28.6	23.3	20.5	18.6	19.3	16.3	15.9	6.6
*Water 4									
Methanol 7									
Ethyl acetate 16	14	6.5	5.4	4.7	3.8	4.0	3.2	3.1	1.1
*Water 16									
Methanol 9									
Ethyl acetate 2	22†	37.9 (0.49)	33.6 (0.38)	29.2 (0.33)	28.0 (0.32)	28.4 (0.32)	26.0 (0.28)	20.9 (0.235)	6.7 (0.025)
Water 2									
Pyridine 1									
Ethyl acetate 3	39†	28 (0.34)	25.3 (0.265)	20.5 (0.22)	17.6 (0.195)	—	14.3 (0.17)	12.4 (0.14)	12.0 (0.13)
Water 3									
Acetic acid 1									
Ethyl acetate 2	14	30.1	26.2	22.4	22.4	21.3	20.8	17.7	5.1
*Water 2									
Dioxan 3									

* These mixtures only just formed two phases, and as the presence of the sugar markedly increased the mutual solubility of the two phases, the spots 'tailed' badly. The separation between the sugar spots was, therefore, less than the figures suggest.

† R_F values are given in parentheses.

In practice the separations achieved are much less than is shown in Table 2, the main reason being that when the solvent commences to drip from the end of the paper, the rate of flow of solvent is reduced to about half the initial value. The relative separations, however, remain substantially the same, though they do not entirely correspond to the R_F values measured when the solvent front is still on the paper. The explanation probably lies in the fact that the distribution of the solvent throughout the paper strip is modified if liquid accumulates at the bottom (cf Consden, Gordon & Martin, 1944).

None of the solvents listed in Table 2 is ideal. One

or two give excellent separations of the pentoses, but do not give an appreciable separation of the hexoses in a convenient time (24 hr). The addition of a third component which is soluble in both phases will tend to increase the solubility of hexoses in the non-aqueous solvent and thereby increase their R_F values. The separation of the pentoses will be relatively less affected. The results obtained with a number of three component mixtures are given in Table 3.

Of these mixtures ethyl acetate-pyridine-water and ethyl acetate-acetic acid-water were selected as they fulfilled the requirements for which this work was undertaken.

Analysis of mixtures of sugars

In a mixture containing arabinose, xylose, glucose, galactose, galacturonic acid and mannose, it was possible to detect all the components with certainty using one or other of the above solvent mixtures. Of these sugars arabinose could not be distinguished from mannose in ethyl acetate-

or by the method described by Hawthorne (1947). We preferred, however, a combination of the two methods. A known amount of the sugar solution was added to the paper from a micropipette (Hawthorne, 1947), and then the sugars separated and isolated following the method described by Hirst *et al* (1947). Analysis of four mixtures by this method is given in Table 4.

Table 4 *Analysis of mixtures of sugars*

	Amount added (μg)	Amount recovered (μg)				
		Individual experiments				Mean
Mixture 1						
Xylose	68.5	61.6	70.4	74.0	67.2	67.0
Arabinose	68.5	77.4	71.3	67.8	67.9	71.1
Mannose	68.5	69.4	76.4	67.3	65.5	69.4
Glucose	68.5	67.9	59.1	77.6	65.3	67.5
Galactose	68.5	74.4	68.9	66.6	71.8	70.4
Mixture 2						
Xylose	68.5	71.0	66.4	75.0	68.1	70.1
Arabinose	68.5	68.0	69.4	74.1	70.4	70.4
Glucose	68.5	75.4	71.1	66.8	64.1	69.3
Galactose	68.5	74.6	61.9	77.1	65.2	69.8
Galacturonic acid	68.5	63.2	85.8	74.2	73.6	74.2
Mixture 3						
Xylose	212	222	215	217	211	216
Arabinose	24.0	25.5	29.2	30.1	20.1	26.4
Glucose	439	433	428	421	426	427
Galacturonic acid	44.3	39.4	46.1	41.8	44.7	43.0
Mixture 4						
Xylose	19.0	19.8	18.4	21.0	23.4	20.7
Arabinose	414	415	391	421	400	406
Glucose	39.8	33.9	45.3	42.1	38.4	39.9
Galacturonic acid	204	208	200	210	214	208

pyridine-water, and galactose and galacturonic acid could not be separated in ethyl acetate-acetic acid-water. If a pair of sugars had very different R_f values separation was completed very rapidly. Thus with ethyl acetate-pyridine-water a mixture of glucose and xylose was completely separated in 2 hr. If only a small proportion of one sugar was present it was advantageous to work with a concentrated solution though the more concentrated the solution used the larger the spot after development. The use of concentrated solutions was possible with the solvents described above because the distance between the centres of the sugar spots was 3–5 cm. Thus, using ethyl acetate-acetic acid-water as solvent, mannose was identified in the presence of 10 times its concentration of glucose and 100 times its concentration of xylose. The total concentration of sugars in the solution used for making the spot was 20% (w/v), and the diameter of the xylose spot after the chromatogram was developed was 4–5 cm.

Quantitative analysis of the sugars on the paper chromatogram after they had been separated using either of these solvents was carried out successfully either by the method described by Hirst *et al* (1947)

Mixture 1 was separated using ethyl acetate-acetic acid-water as solvent. The time required for the separation of the sugars was 48 hr. Mixtures 2, 3, 4 were separated using ethyl acetate-pyridine-water as solvent. In the case of mixtures 3 and 4 the composition of the solvent was slightly modified, the components being in the ratio of 5, 2, 5 instead of the ratio of 2, 1, 2 respectively as described in Table 3. The modified solvent gave slightly better separations than the original. The papers were dried at room temperature overnight because it was found that drying at the usual temperature of 60–100° in the presence of pyridine appeared to destroy a large part of the sugar present.

Mixtures 3 and 4 are examples of the separations achieved when some of the sugars are present only in small proportions.

DISCUSSION

If we consider the separation of the sugars after they have moved a definite distance down the paper chromatogram we find that the best separations are obtained with solvents which give R_f values less

than 0.2, the solvent being allowed to run off the end of the paper. This observation is in agreement with predictions which can be made by applying to the paper chromatogram the equation first deduced by Martin & Synge (1941) for the relation between the complete separation of two solutes (99.8% of one and 0.2% of the other) and the characteristics of the partition column

$$\frac{A_L + \alpha A_S}{A_L + \beta A_S} = \frac{r - 3\sqrt{r + 2.5}}{r + 3\sqrt{r + 2.5}}$$

where α and β are the partition coefficients of the two solutes, A_L is the area of cross-section of the mobile phase, A_S is the area of cross-section of the non-mobile phase and r represents the number of 'theoretical plates' the first solute has passed. At any particular place in the column $\frac{r - 3\sqrt{r + 2.5}}{r + 3\sqrt{r + 2.5}}$ is

a constant and represents the maximum value that $(A_L + \alpha A_S)/(A_L + \beta A_S)$ can have if the two solutes are to be just separated. If A_L is small in relation to αA_S or βA_S the expression reduces to α/β . This is true when the partition coefficients are large. Now if we assume that the ratio α/β is likely to be sensibly constant for solvents of a similar type it follows that $(A_L + \alpha A_S)/(A_L + \beta A_S)$ reaches its minimum value when the partition coefficients are large, and that if two solutes are only just separated when the partition coefficients are large they will not be separated when the partition coefficients are small. The assumption that the ratio α/β is sensibly constant for solvents of a similar type is strongly suggested by analogy with the data given by Cohn & Edsall (1943) on the solubility of amino-acids and their derivatives in various organic solvents and in water. It is important, therefore, to choose a solvent for which the partition coefficients are large so that the maximum separation is achieved. In paper chromatograms A_L/A_S is about 4 (Consden *et al.* 1944) so that α and β must be at least 10–15. This corresponds to an R_F value of 0.2–0.3.

In practice it is not always easy to find a two-component solvent (water is always one component) which will give R_F values of about 0.2 for a particular pair of sugars. Three component solvents are, however, much more flexible in this respect. Originally these solvents were introduced in order to obtain a more convenient spread of R_F values for pentoses and hexoses than was possible with two component solvents, but they can be readily applied to the separation of sugars with similar R_F values. The R_F value of a sugar can be altered by changing the amount of water dissolved in the organic phase, the general relationship between the amount of water in the organic phase and the R_F value is clearly shown by the figures in Table 1. The addition to a

two-component system of a third substance, which is readily soluble in both phases, increases the amount of water held in the non aqueous phase, whereas addition of a substance which is soluble only in one phase diminishes the amount of water. Thus, by using the organic phase from a mixture consisting of two organic solvents and water, one of the organic solvents being miscible with water and the other immiscible, it is possible to alter the R_F value within wide limits. The R_F value for any particular pair of sugars can, therefore, be adjusted to about 0.2.

The apparatus used in the present experiments has the advantage that during the development of the chromatogram the phases on the paper are not disturbed. In the method described by Partridge (1948) the lid of the chamber is removed when the trough is filled with liquid, and the atmosphere is, therefore, initially unsaturated, and requires several hours at least to reach equilibrium with the solvent. During this time the advancing solvent front is losing solvent to the atmosphere, and particularly in the case of three component systems may not have the same composition as the solvent in the trough. In extreme cases with certain mixtures the two phases become miscible and then all the sugars travel down the paper just behind the solvent front. By filling the trough without opening the chamber after the paper and atmosphere have reached equilibrium with the solvent, we can avoid any disturbance of the phases on the paper.

SUMMARY

1 A number of solvents have been examined for the separation of complex sugar mixtures on the paper chromatogram. It has been shown that two three-component mixtures, ethyl acetate-acetic acid-water and ethyl acetate-pyridine-water are more satisfactory than any previously described solvents for the qualitative and quantitative determination of the sugars in mixtures of the simple hexoses and pentoses.

2 Some of the factors which influence the separation of the sugars on the paper chromatogram are described and analyzed. It is shown that improved separations can be obtained by using solvents which give low R_F values, the solvent front being allowed to run off the paper. The use of a solvent with a low viscosity shortens the time needed for analysis because the solvent flows more rapidly through the paper.

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Zinc Uptake by *Neurospora*

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Among the minor constituents of living tissues zinc is one of great interest (see Lehman, 1939, Broh-Kahn & Mirsky, 1948, Warburg, 1948, Bertrand, 1948) It constitutes 0.33% of carbonic anhydrase (Keilmann & Mann, 1940), and it is a constituent of uricase which contains 1.3% of zinc (Davidson, 1938, Holmberg, 1939) and possibly of other enzymes It was found to be essential for growth and citric acid formation of *Aspergillus niger* (Bernhauer, 1928), for growth and fumaric acid production of *Rhizopus nigricans* (Foster & Waksman, 1939) and for growth and subtilin formation by *Bacillus subtilis* (Feeney, Lightbody & Garibaldi, 1947)

The presence of 0.2 mg of zinc in the food of mice is stated by Bertrand (1948) to prolong the life of the animals from 2–3 weeks to 2–2.5 months

Wassiljew (1935) demonstrated that the effect of zinc was not the same for biochemically different strains of *A. niger*, and he went so far as to suggest a characterization of *A. niger* strains based on their reactivity to zinc Foster & Waksman (1939) interpret the function of zinc as catalyzing a more complete destruction of the glucose molecule with a consequent greater efficiency of energy utilization by the fungus

In connexion with studies on the growth of *Neurospora crassa* we determined the amount of zinc necessary to obtain a maximum growth of that mould

METHODS

Throughout the experiments mycelium from one spore was used This was produced by crossing the two wild type strains, nos 25a and 1A of Beadle (1945) The culture solution was that described by Ryan, Beadle & Tatum (1943), it was autoclaved, and the cultures were treated under sterile conditions Inocula were taken from cultures 4–5 days old

Labelled zinc as chloride and varying amounts of non-radioactive zinc as sulphate were added to culture solutions at the start of the experiments From the specific activities of the zinc present in the culture solution and in the mould, and the amount of zinc added to the culture solution, the zinc content of *Neurospora crassa* was calculated We are much indebted to the Atomic Energy Commission of the USA and the Chief of the Isotope Branch, Dr Poul Aebersold, for supplying the zinc containing the radioactive isotope ^{65}Zn

The activity of the ^{65}Zn taken up by *Neurospora* was less than 1 μcurie , and thus the radiation emitted could hardly produce any noxious action on the mould

After centrifuging and thorough washing the *Neurospora* was dried at 105°, brought into solution by wet ashing, 5 mg zinc as sulphate added as carrier, and the zinc precipitated with 8-hydroxyquinoline The precipitate was filtered through a perforated aluminium dish covered by a layer of filter paper, and the dish placed under a Geiger-Müller counter of the type described by Madsen (1945) A sample of the active ZnCl_2 solution was treated in the same way By comparing the activities of this 'standard' precipitate of known zinc content and that of the above-mentioned precipitates, the percentage of the zinc originally present in the culture solution which accumulated in the mould was calculated

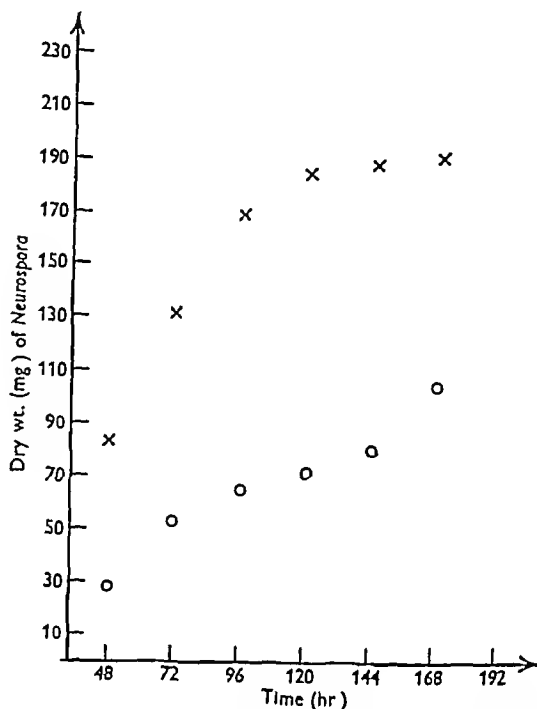
RESULTS

The results of some of our experiments, in which each *Neurospora* was grown in 150 ml culture solution of varying zinc content for 5 days at 25°, are given in Table 1 This table and Fig 1 show that the presence of 1 part of zinc in about 30,000 parts of dry mould is sufficient for an almost maximal growth, and the presence of 12.5 μg zinc in 150 ml. culture solution suffices to give that zinc content It is advisable to add a somewhat larger amount of zinc to the culture solution, e.g. 200 μg / l as recommended by Ryan *et al* (1943) or 450 μg / l as stated by Mitchell & Houlakan (1946)

Table 1 Amount of zinc taken up by *Neurospora*

Zinc in culture solution (μg)	Wt of mould (mg)	Zinc (μg /mg dry mould)	Percentage of the ^{65}Zn content of culture solution present in 1 mg dry mould
2.5	106.6	0.021	0.84
	106.9	0.013	0.50
	97.8	0.012	0.48
	90.0	0.014	0.55
Average	100.3	0.015	0.59
12.5	175.2	0.033	0.26
	169.7	0.033	0.26
	151.7	0.035	0.28
	144.3	0.040	0.32
Average	158.9	0.035	0.28
50.0	208.6	0.090	0.18
	178.8	0.11	0.21
	168.1	0.10	0.19
	166.7	0.11	0.22
Average	180.5	0.10	0.20
250.0	153.8	0.13	0.033
	172.3	0.12	0.043
	147.7	0.11	0.044
	161.9	0.14	0.055
Average	158.9	0.13	0.049
2000.0	186.3	0.30	0.015
	184.5	0.34	0.017
	158.3	0.38	0.019
	164.0	0.38	0.019
Average	168.3	0.35	0.018

weight was still more pronounced (Fig 2) After 48 hr 28 mg were obtained in the absence of added zinc, while adding 30 μg of zinc to a 150 ml culture produced 83 mg of mould

Fig 2 Dry weight of *Neurospora* without (O) and after (X) addition of 30 μg of zinc

After 5 hr, a 150 ml culture solution containing 2.5 μg zinc produced 100.3 mg and one with 12.5 μg zinc, an almost maximal weight of 158.9 mg. In the former case 1 mg *Neurospora* contained 0.015 μg zinc, in the latter roughly twice that amount (0.035 μg). An increase in the zinc content of the culture solution to 2000 μg did not increase the weight of *Neurospora* observed after the lapse of 5 days (Table 1). The capacity of the fungus to take up zinc was, however, not exhausted, as shown by the fact that an eightfold increase in the zinc concentration of the solution resulted in an almost threefold zinc uptake by the mould.

Feeney *et al* (1947) determined the amount of zinc to be added to the culture solution in order to obtain maximal growth and maximal antibiotic formation in cultures of *Bacillus subtilis*. This was found to be 0.40 mg/l, while in the case of *Neurospora crassa* an almost maximal growth was observed by us in the presence of as little as 0.08 mg/l of zinc in the culture solution.

Migration of zinc from the *Neurospora* into the culture solution

By making use of isotopic indicators the migration of ions from the plant into the culture solution has been observed in several cases, and the effect on the

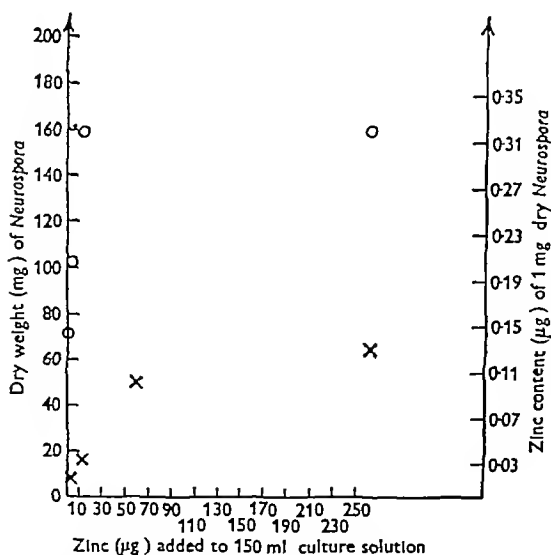
Fig 1 Effect of varying zinc content of the culture solution on the dry weight (O) and zinc content (X) of *Neurospora* after the lapse of 5 days

Fig 1 shows the very pronounced increase in weight of the mould, from 71 to 100 and 159 mg, after the addition of 2.5 and 12.5 mg of zinc respectively to the culture solution. In experiments of shorter duration the effect of zinc on the increase in

migration process of unlabelled ions in the solution has also been studied. Minute amounts of labelled lead, when taken up by the roots of *Vicia Faba*, could to a large extent be removed by an excess of non-labelled lead added to the nutrient solution, but to a minor extent only if the solution did not contain lead (Hevesy, 1923).

Mullins & Brooks (1939) placed cells of *Nitella coronata* first in a solution containing radioactive potassium (^{42}K) and later in solutions of different chlorides. Sodium and lithium were found to be much less effective in removing labelled potassium than potassium itself. We investigated the exodus of labelled zinc into the culture solution both in the absence and presence of zinc in the solution in which the mould containing labelled zinc was placed.

In these experiments *Neurospora* grown for 5 days at 28°, after repeated washing with distilled water, was divided into five approximately equal parts. One part was kept as a control, two parts immersed separately in 150 ml culture solution containing no zinc for 24 hr at 12°, while two parts were kept under similar conditions in a culture solution containing 30 µg zinc.

Table 2 *Migration of labelled zinc from Neurospora into the culture solution*

Sample	Activity
Control	100
<i>Neurospora</i> after being kept in zinc free solution for 24 hr at 12°	88, 84
<i>Neurospora</i> after being kept in zinc containing solution for 24 hr at 12°	74, 75
Zinc free solution after 24 hr	9, 10
Zinc containing solution after 24 hr	14, 19
Penultimate wash water	1

In all our experiments more labelled zinc left the mould when placed in a solution containing zinc. Table 2 shows the results of a typical experiment.

Uptake of cobalt by Neurospora

We compared the uptake of Co^{++} (using ^{58}Co as an indicator) with that of zinc by *Neurospora*. Four moulds were grown for 5 days at 28° in the presence of 25 µg of cobalt in the culture solution. They were found to take up 0.013, 0.011, 0.011 and 0.011 % of the cobalt content of the solution per mg of their dry weight (i.e. 0.0033, 0.0028, 0.0028 and 0.0028 µg). The cobalt uptake corresponds to only about one-twentieth of the uptake of zinc under similar conditions, showing that the moulds do not possess such an effective mechanism for fixing cobalt as they do for zinc.

SUMMARY

1 By making use of labelled zinc (^{65}Zn) the amount of zinc necessary to obtain maximal growth of *Neurospora crassa* was studied.

2 Maximal growth requires that at least 1/30,000 part of the dry weight of the *Neurospora* be composed of zinc, and can be obtained by raising the mould in a culture solution containing 0.08 mg or more of zinc/l.

3 When placed in a culture solution containing inactive zinc more labelled zinc migrates from *Neurospora* into the solution at 12° than in the absence of zinc.

4 Cobalt uptake, studied by making use of labelled cobalt (^{58}Co), amounts only to one twentieth of the uptake of zinc under corresponding conditions.

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Accumulation of Glutamic Acid in Isolated Brain Tissue

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Van Slyke (1913) was the first to show that most animal tissues have a far higher concentration of free amino acids than has blood plasma. For example, 75, 73 and 64 mg non-protein amino N were found in 100 g of dog liver, kidney and spleen respectively, and only about 4–5 mg in 100 g of blood plasma (Van Slyke & Meyer, 1913). Hamilton (1945) and Krebs, Eggleston & Hems (1949) have reported comparable differences for the distribution of glutamine and glutamic acid between tissues and plasma. Since amino acids can travel readily from tissue to plasma, and plasma to tissue, special mechanisms must exist which control the rate of transport and maintain high concentration gradients.

This paper is concerned with a study of this mechanism. When slices of brain and other tissues are kept in a saline medium containing glutamate the concentration of this amino-acid in the tissue rises under some conditions and falls under others. Transport of glutamate can occur against a considerable concentration gradient. The changes observed are sufficiently large to allow accurate measurements, and the factors which control the concentration gradient between tissue and medium can thus be investigated under relatively simple conditions.

EXPERIMENTAL

Handling of tissue. In the present experiments it was necessary to measure the fresh weight of the tissue before incubation in a nutrient medium. It was therefore essential to avoid contact of the tissue with medium during the slicing operations. After preliminary experiments the following procedure was adopted. The brain of a guinea pig was removed from the animal immediately after death and placed on dry filter paper for slicing. Slices of the grey matter were made with a dry cutting blade by the method of Deutsch (1936), or of Stadie & Riggs (1944), and were weighed on a torsion balance. Samples of c. 0.15 g fresh weight were placed in the medium in the main compartment of each conical Warburg cup, in which they were shaken at 40° for specified periods. At the end of the incubation the cups were placed in ice water to stop metabolic activity and the slices were collected as quickly as possible. After draining off the medium along the wall of the manometer vessel the slices were again weighed on the torsion balance. They were then ground in a mortar with 2.5 ml of 0.4N-HCl. The remaining medium was acidified with 0.25 vol of 2N HCl immediately after the removal of the

tissue and ice cooled. The sum of L-glutamic acid and L-glutamine was determined in a measured fraction of this solution and in 2 ml of the suspension of ground tissue. In each experiment a sample of slices which had not been incubated was treated and analyzed in the same way as the incubated material.

Rabbit brain was found to be less suitable because slices of this material broke up into many particles when shaken in saline media, whilst guinea pig brain slices remained coherent. Smaller animals, such as rats or pigeons, did not yield enough slices for a series of experiments on one brain. One guinea pig brain was usually sufficient for five samples of 0.15 g each.

All data on tissue weights given in this paper refer to fresh weights.

Medium. Phosphate saline without Ca (Krebs & Eggleston, 1940) was used as the standard medium. Substrates were added in the form of neutral 0.2M solutions and the total volume of the medium, including the added substrates, was 4 ml. The substrates were added to the medium from the side arm immediately before the manometers were attached to the water bath. Unless otherwise stated, the gas space of the vessels was O₂.

Analytical methods. The decarboxylase method of Gale (1945), as modified by Krebs (1948), was used for the determination of the sum of L-glutamic acid and L-glutamine. It is specific for these two substances. D-Glutamate is not attacked by the bacterial decarboxylase. As a rule no effort was made to differentiate between the two substances and in the following tables 'glutamic acid' or 'glutamate' refer to the sum of L-glutamic acid and L-glutamine except where it is specifically stated that a differential determination was carried out.

Calculation. A difficulty in the quantitative interpretation of the measurements arose from the finding that the thoroughly drained brain slices collected at the end of the incubation always weighed more than the fresh slices as placed in the medium. The increase was usually about 25–50% under aerobic, and 50–75% under anaerobic, conditions, and could have been due either to the adherence of saline medium to the tissue or to swelling of the latter. Elliott (1946) has in fact shown that brain tissue swells when immersed in saline media and this is confirmed by observations reported in this paper, however, the weight increase observed under our conditions was probably due to both swelling and wetting.

If the concentration of glutamate in the slices is calculated on the assumption that the weight increase is due to adherent medium, higher values are obtained than if it is assumed to be due to swelling. Examples showing the extent of the differences obtained by the two methods of calculation are given in Tables 1 and 2. In contrast, the figure for

the total amount of glutamate taken up by the slices is not much affected by the method of calculation as long as the concentration of glutamate in the medium is much lower than in the slices, which was the case in most experiments. To what extent the calculated results vary with the concentration of glutamate in the medium can be seen from the data given in columns 3 and 5 of Table 2

In all cases, except in the specified columns of Tables 1 and 2, the amount of glutamic acid in the slices was calculated on the assumption that all the weight increase was due to adherent medium. The concentration of glutamic acid in the medium at the end of the incubation period was determined in every experiment so that the correction for the adherent medium could be made. Any errors arising from the incorrectness of the assumption would cause the uptake of glutamate by the tissue to appear too small, and the concentration of glutamate in the slices to appear too high.

The amount of glutamic acid removed from the medium was in most experiments larger than the amount which accumulated in the tissue. This is taken as an indication that the tissue had converted some glutamic acid into other substances. This fraction of glutamic acid (glutamic acid removed from medium minus glutamic acid accumulated in tissue) will be referred to as 'glutamic acid metabolized', in accordance with general usage, the removal of glutamic acid from the system will be denoted by the minus sign. In some cases, indicated by the positive sign, glutamic acid in the system 'medium plus slices' increased. Where the determination of 'glutamic acid metabolized' rests on the measurement of a difference, in the presence of a total amount of c. 400-900 μ l glutamate, small values for glutamic acid metabolized (below 20 μ l) are of doubtful significance (1 μ mol glutamic acid \equiv 22.4 μ l, cf Krebs, 1948).

RESULTS

Accumulation of L glutamic acid in slices of brain cortex. On incubation with glutamate, in the presence of glucose, the amount of glutamate in the slices increased progressively during the first 60 min up to about two and a half times the initial value (Table 1, cols 4 and 5). At the same time glutamate disappeared from the medium (Table 1, cols 8 and 9). The differences in the figures given in

columns 5 and 9 show that, except for the first 20 min period, where the measurement was not very accurate owing to the relatively small change, considerable amounts of glutamate were metabolized under the conditions of the experiment.

The transport of glutamate into the tissue occurred against a concentration gradient (see Table 1, cols 10-12). The computations of this gradient give somewhat, but not decisively, different figures according to the assumptions on which they rest (cf Table 1, cols 10 and 11).

During the 60-80 min period the glutamic acid concentration dropped in the slices whilst it increased in the medium. Thus the mechanism of transporting glutamic acid into the tissue or of maintaining the concentration gradient had lost its efficiency after 60 min, presumably owing to tissue damage. In later experiments on brain tissue where rates were to be measured, the incubation period was usually 40 min.

A comparison of the rate of accumulation of glutamate in brain tissue with the metabolic processes shows that the average rate of accumulation in the slice was 3.7 μ l glutamic acid/mg dry wt/hr for the first 20 min and 3.1 for the first 60 min. The average rate of removal from the solution, on the basis of the 60 min value, was 4.9. Thus the rate of glutamate removal by metabolic reactions was 1.8, or about one third of the rate of removal from the solution. For the purpose of these calculations it was assumed that the dry weight of the non-incubated slices was 20% of the wet weight.

Effect of the concentration of L-glutamate. Increasing the concentration of glutamate in the medium caused an increased uptake by the slices (Table 2, cols 4 and 6). It is remarkable that at the end of the incubation period the differences between the concentrations in the slices and in the medium were about the same in spite of great differences at the start (see Table 3). The amounts of glutamate

Table 1. Accumulation of L glutamate in slices of guinea pig brain cortex

(0.02M Glucose, 0.005M L glutamate. The values given in column 10 were calculated by dividing the corresponding figures in col. 4 by those in col. 2, the values in col. 11 by dividing the figures in col. 6 by those in col. 1.)

Period of incuba tion (min)	Wt of slices/cup		Wt increase of slices (g)	L Glutamate found in drained slices		L Glutamate in slices corrected for adherent medium		L-Glutamate in medium		Concentration of L glutamate		
	Initial (g)	After incuba tion (g)		Total (μ l)	Change (μ l.)	Total (μ l)	Change (μ l)	Total found (μ l)	Change (μ l)	In slices		
										As weighed (μ l/g)	Corrected for adherent medium (μ l/g)	In medium (μ l/ml.)
Column	1	2	3	4	5	6	7	8	9	10	11	12
0	0.146	—	—	60	—	60	—	428	—	412	412	107
20	0.145	0.210	+0.065	96	+36	91	+31	398	-30	458	628	100
40	0.143	0.217	+0.074	117	+57	112	+52	327	-101	540	781	82
60	0.148	0.226	+0.078	151	+91	146	+86	282	-146	666	986	71
80	0.149	0.219	+0.070	122	+62	117	+57	292	-136	555	785	73

Table 2 *Effect of L-glutamate concentration on the accumulation in slices of brain cortex*(Period of incubation 40 min, 0.02M glucose, initial amount of L glutamate in 150 mg slices, 49 μ l)

Initial amount of L glutamate in 4 ml (μ l)	Wt of slices/cup		L Glutamate found in drained slices		L Glutamate in slices corrected for adherent medium		L Glutamate found in 4 ml medium after incubation		Final concentration of L glutamate		
	Initial (g)	After incubation (g)	Total (μ l)		Total (μ l)		Total (μ l)		In slices		
									Corrected for adherent medium		
									As weighed (μ l/g)	Corrected for adherent medium (μ l/g)	In medium (μ l/ml)
Column	1	2	3	4	5	6	7	8	9	10	11
222	0.149	0.224	85	+ 36	84	+35	116	- 106	382	561	29
444	0.149	0.221	92	+ 43	86	+37	361	- 83	414	580	90
888	0.144	0.206	114	+ 67	105	+58	733	- 155	552	726	183
1776	0.147	0.218	157	+109	134	+86	1607	- 169	720	912	402

metabolized (calculated from columns 8 and 4 or 6 of Table 2) did not vary much with the glutamate concentration in the medium. This is not unexpected as the metabolic reactions should depend on the substrate concentration in the tissue, which showed relatively little variation, rather than on the substrate concentration in the medium.

Table 3 *Difference in the concentration of L glutamate between slices and medium*

(Calculated from data given in Table 2. For the purpose of the calculation it is assumed that 1 g slice equals 1 ml slice. 'Difference' is concentration in slices minus concentration in medium. The minus sign indicates that the concentration in the slices was lower than the concentration in the medium.)

Initial difference (μ l/ml)	Difference after incubation	
	Calculated from cols 9 and 11 (μ l/ml)	Calculated from cols 10 and 11 (μ l/ml)
282	353	532
216	324	490
115	368	540
- 111	318	510

Effect of various substrates on the fate of L glutamate

When L glutamate was the only substrate added to brain slices the original concentration of glutamate in the tissue was not maintained but fell (Table 4, Exp 1). Succinate, fumarate, citrate, α glycerophosphate, phosphoglycerate or adenosine triphosphate were unable to prevent this fall. In the presence of glucose, fructose, pyruvate, lactate and fructose 1,6 diphosphate the concentration of glutamate rose on incubation. The effect of glucose was greater than that of the other substances, except in one case (Exp 1) where the effect of glucose was unusually low and where lactate was more effective than glucose.

A comparison of the effects of the above substances with their oxidizability in brain tissue shows no simple correlation. Substances which cause an

accumulation of glutamate in the tissue are all oxidizable, but oxidizability alone is not sufficient to support accumulation, glutamate, succinate and α -glycerophosphate, although readily oxidized, were ineffective even in preventing the fall of glutamate in the tissue. Whilst the oxygen uptakes in the presence of glucose, lactate and pyruvate were of the same order, glucose was definitely more efficient in supporting accumulation.

The amount of glutamate metabolized was by far the greatest in the presence of oxaloacetate, transamination being mainly responsible for the removal in this special case (see Exp 3). The relatively large amount of glutamate metabolized in the presence of fumarate was probably also due to transamination with the oxaloacetate arising by oxidation of the fumarate. In general, substrates which were readily oxidized, like glucose, lactate or pyruvate, reduced the amount of glutamate metabolized, whilst substances which are not readily oxidized in brain tissue had no appreciable effect.

The rate of glutamate removal in the presence of oxaloacetate, in terms of the usual Q values (μ l/mg dry wt/hr), was 28, and if the reaction had occurred within the brain cells this figure would represent the minimum rate of entry of glutamate into the tissue. On the other hand, the data in Table 1 indicate an actual rate of entry under similar conditions of no more than 5. To clear up this apparent discrepancy experiments were carried out to test whether the transaminase diffuses into the medium and allows the reaction to take place outside the slice. Brain slices (138 mg) were shaken in glucose saline (4 ml) for 40 min in an atmosphere of O_2 . The slices were then removed, the medium was centrifuged and 3 ml of the supernatant were incubated at 40° with 0.2 ml of 0.02M-glutamate, 0.4 ml of 0.02M oxaloacetate and 0.4 ml of saline. After 40 min the reaction was stopped by the addition of 2N-HCl (1 ml) and glutamate was determined in the solution. Of the 890 μ l glutamate added only 244 were recovered. It

Table 4 *Effect of various substrates on the fate of L-glutamate in guinea-pig brain cortex*

(Incubation 40 min Concentration of glutamate 0.01 M, of other substrates 0.02 M unless otherwise stated)

Exp no	Substrates added in addition to glutamate	Initial wet wt of slices (g)	Amount of glutamate found in slices			Amount of glutamate removed from medium (μ l)	Amount of glutamate metabolized (μ l)
			Initially (μ l)	After incubation			
				Total (μ l)	Change (μ l)		
1	None	0.162	59	52	- 7	- 54	- 61
	Glucose	0.165	60	100	+40	- 86	- 46
	Pyruvate	0.160	58	91	+33	- 63	- 30
	L Lactate	0.168	61	112	+51	- 68	- 17
2	Glucose	0.144	52	148	+96	-208	-112
	Fructose	0.185	67	124	+57	-125	- 68
	Pyruvate	0.167	60	103	+43	-127	- 84
	L Lactate	0.157	57	101	+44	- 93	- 49
3	Glucose	0.157	55	127	+72	-144	- 72
	Oxaloacetate	0.151	53	57	+ 4	-566	-562
	α Glycerophosphate	0.194	67	50	-17	- 73	- 90
	Adenosinetriphosphate (0.002M)	0.162	56	42	-14	- 90	-104
4	Glucose	0.167	65	160	+95	-126	- 31
	Succinate	0.161	63	51	-12	- 73	- 85
	Fumarate	0.169	66	56	-10	-130	-141
	Citrate	0.169	66	42	-24	- 85	-109
5	Glucose	0.153	53	120	+67	-155	- 98
	DL Phosphoglycerate	0.143	49	42	- 7	- 25	- 32
	Adenosinetriphosphate (0.002M), DL-phosphoglycerate	0.159	55	57	+ 2	- 59	- 57
	Fructose 1.6 diphosphate	0.197	68	95	+27	- 66	- 39

follows that enough transaminase can diffuse out of the tissue slices to account for the change in glutamate concentration observed in Exp 3 of Table 4. Thus whilst the slices can retain and even concentrate glutamate they lose the very much larger molecule of the transaminase whose molecular weight has been estimated at 60,000 (Green, Leloir & Nocito, 1945). Further quantitative assays of the glutamic aspartic transaminase showed that after 40 min incubation in glucose saline 25% of the enzyme had passed from brain slices into the medium. Whipple & Madden (1944) have adduced evidence which suggests that other large molecules—

the plasma proteins—flow readily to and from between tissue and plasma.

Comparison of L-glutamate and L glutamine After a short period of incubation (15 min) the amount of glutamate plus glutamine found in the slices was greater when glutamine was the substrate, whilst after 30 min the concentrations reached with glutamate and glutamine were about equal (Table 5). The data suggest that glutamine is taken up a little more rapidly than is glutamate. In a similar experiment a differential determination of glutamate and glutamine was carried out after an incubation period of 60 min. The method recently described was used.

Table 5 *Comparison of the rates of accumulation of L-glutamate and L-glutamine in guinea-pig brain cortex*

(Glucose 0.02 M)

Exp no	Period of incubation (min)	Initial wt of slices (g)	Substrates added (0.01 M)	Sum of glutamate and glutamine found in slices	
				Total (μl)	Increase (μl)
1	0	0.161	—	56	—
	15	0.166	Glutamate	75	19
	15	0.166	Glutamine	97	41
	30	0.165	Glutamate	96	40
	30	0.167	Glutamine	115	59
2	0	0.160	—	62	—
	15	0.155	Glutamate	80	18
	15	0.157	Glutamine	107	45
	30	0.163	Glutamate	131	69
	30	0.154	Glutamine	124	62

(Krebs, 1948) When glutamate was the substrate in the medium over 90 % of the total glutamate in the tissue was present as glutamic acid, whilst with glutamine as substrate 45 % of the total glutamate in the slice was present as glutamine. Hence both forms can enter the tissue.

Anaerobic experiments Anaerobically glucose partly prevented the fall in the concentration of glutamate which occurred when glutamate or glutamine were the only substrates in the medium (Table 6), and in some experiments (Exp. 3) there

glycolysis by glutamate, which was previously unexplained, and for the abolition of the inhibition by adenosinetriphosphate.

Effects of inhibitors Two kinds of effect, that on the concentration of glutamate in the tissue and that on the metabolic utilization of glutamate, were investigated. Guinea-pig brain cortex served as material and in all experiments the medium contained 0.02M-glucose and 0.01M L-glutamate. The period of incubation was 40 min. DL-Methionine sulfoxide (0.02M), sodium penicillin (0.1 %) and

Table 6. Accumulation of L glutamate in slices of brain cortex under anaerobic conditions

(0.02M Glucose, 0.01M glutamate or glutamine, 0.002M adenosinetriphosphate, 0.02M DL phosphoglycerate. Bicar bonate saline used in Exp. 1, phosphate saline in the other experiments. Incubation 30 min in Exps. 1, 3 and 4, 40 min in Exp. 2.)

Exp no	Substrate added	Initial wt of slices (g)	Amount of glutamate found in slices		
			Initial (μ l)	After incubation	
				Total (μ l)	Change (μ l)
1	Glutamate, glucose	0.147	42	33	-9
	Glutamate, glucose, adenosinetriphosphate, phosphoglycerate	0.155	44	38	-6
	Glutamate, glucose, adenosinetriphosphate	0.161	46	38	-8
	Glutamate, glucose, phosphoglycerate	0.145	41	41	0
2	Glutamate	0.101	42	15	-27
	Glutamine	0.100	42	21	-21
	Glutamine, glucose	0.101	42	28	-14
	Glutamine, adenosinetriphosphate	0.107	45	21	-24
	Glutamine, glucose, adenosinetriphosphate	0.100	42	27	-15
3	Glutamate, glucose	0.158	48	53	+5
	Glutamine, glucose	0.163	50	69	+19
4	Glutamate, glucose	0.164	58	46	-12
	Glutamine, glucose	0.168	59	61	+2

was even a small increase of the tissue glutamate in the presence of glucose. This increase was greater with glutamine than with glutamate in the medium. Adenosinetriphosphate and phosphoglycerate had no appreciable effect on the accumulation of glutamate. In these experiments glycolysis was also measured and it was noted that adenosinetriphosphate removed the glutamate inhibition (Weil Malherbe, 1938) of glycolysis. In Exp. 1 of Table 6, for example, $Q_{\text{lactic acid}}$ was 6.4 in the presence of glucose and glutamate and 16.0 when adenosinetriphosphate was also added. The fact that addition of adenosinetriphosphate accelerates glycolysis may be taken to indicate that, in the presence of glutamate, adenosinetriphosphate is a limiting factor in glycolysis and it would follow that glutamate reduces the concentration of adenosinetriphosphate. This might be the result of the formation of glutamyl- γ -phosphate, a reaction postulated by Elliott (1948a, b) as a step in the synthesis of glutamine in brain. Addition of adenosinetriphosphate would overcome the inhibition of glycolysis by restoring the original concentration of this substance in the tissue. This hypothesis accounts for the inhibition of

strychnine (0.0025M) had no effect. Sodium fluoride (0.02M) and phlorrhizin (0.01M) caused a fall in the tissue concentration of glutamate and in its utilization. Malonate (0.02M) reduced accumulation a little and inhibited almost completely the metabolic utilization. In the presence of crystal violet (0.01 %), 2,4-dinitrophenol (0.001M) and iodoacetate (0.001M) the utilization was not affected, but the accumulation was reduced in that the tissue concentration remained almost stationary, i.e. no fall occurred and no storage beyond the initial level.

To test whether D-glutamate has inhibitory effects DL- and L-glutamate were compared. Whilst accumulation of L-glutamate in the tissue was practically unaltered in the presence of DL-glutamate, much less, if any, L-glutamate appeared to have been metabolized. However, an analysis of this effect suggested that the apparent inhibition of L-glutamate removal by D-glutamate was due to a conversion of D-glutamate into L-glutamate by brain tissue. For example, on incubation (40 min) of 155 mg slices (fresh weight) with 0.01M-D-glutamate and 0.02M glucose, an increase of 12 μ l of L-glutamate was found in the slices and of 38 μ l in the

medium No increase occurred in the absence of D-glutamate and it is therefore very probable that the L-glutamate formed had arisen from D-glutamate Weil-Malherbe (1936) and Edlbacher & Wiss (1944) have shown that brain tissue can deaminate D-amino-acids, and deamination is presumably the primary step in the conversion of the D- into the L-form This view is supported by the observation that α -ketoglutarate and ammonia yield L-glutamate in brain slices at a sufficient rate (Weil-Malherbe, 1936)

Accumulation of glutamate in tissues other than brain Experiments similar to those on brain were carried out on nine other tissues (Table 7) Six of these, when suspended in saline containing L-glutamate and glucose, showed no increase in their

glutamate concentration unless the concentration of glutamate in the medium was higher than the original concentration in the tissue If a substrate for respiration was present, however, these tissues roughly maintained their original concentration of glutamate even when no glutamate was added to the medium The six tissues were testis (rat), kidney medulla (guinea pig), liver (guinea pig), wall of the small intestine (rat), choroid plexus (sheep) and amnion (guinea pig) Records of the experiments on these tissues are not included in Table 7 except for one on guinea-pig liver It was thought that the failure of this tissue to accumulate glutamate might possibly be due to the relatively low rate of penetration of glutamate (see Krebs, Eggleston & Hems, 1948), but this was not confirmed by the results of

Table 7 *Accumulation of L-glutamate by various tissues*

(Initial concentration of added substrates 0.02M except where otherwise stated)

Tissue	Substrates added	Period of incubation (min)	Weight of tissue (g)	Glutamate found in tissue (μ l)	Change of glutamate in medium (μ l)	Glutamate metabolized (μ l)	Final concentration of glutamate	
							Tissue (μ l/g)	Medium (μ l/ml)
Kidney cortex, guinea pig	None	0	0.153	18	—	—	—	—
	None	60	0.152	11	+ 27	+ 20	72	7
	Glutamate (0.01M)	60	0.146	61	- 137	- 93	416	190
	Glutamate (0.01M), glucose	60	0.153	63	- 170	- 125	412	182
Kidney cortex, guinea pig	None	0	0.131	19	—	—	—	—
	None	30	0.140	10	+ 23	+ 14	72	6
	Glutamate (0.01M), glucose	30	0.155	44	- 94	- 69	283	188
Kidney cortex, guinea pig	None	0	0.154	22	—	—	—	—
	Glutamate (0.01M)	40	0.148	46	- 94	- 70	313	189
	Glutamate (0.01M), glucose	40	0.150	45	- 84	- 59	298	191
	Glutamate (0.01M), L lactate	40	0.147	38	- 52	- 36	258	199
Lung, guinea pig	None	0	0.180	23	—	—	—	—
	None	40	0.160	19	+ 21	+ 20	120	5
	Glutamate (0.003M)	40	0.164	24	- 33	- 31	147	70
	Glutamate (0.003M), glucose	40	0.173	22	- 22	- 22	127	73
	Glucose	40	0.161	15	- 10	+ 7	93	3
Lung, guinea pig	None	0	0.139	16	—	—	—	—
	None	40	0.151	12	+ 14	+ 10	80	3
	Glutamate (0.005M), glucose	40	0.151	33	+ 40	+ 25	219	109
Spleen, guinea pig	None	0	0.103	7	—	—	—	—
	None	40	0.092	13	+ 14	+ 6	140	4
	Glutamate (0.003M)	40	0.104	20	- 17	- 4	192	74
	Glutamate (0.003M), glucose	40	0.112	20	- 14	- 3	178	75
	Glucose	40	0.106	22	- 23	+ 9	207	6
Liver, guinea pig	None	0	0.159	33	—	—	—	—
	None	60	0.161	10	+ 63	+ 40	62	16
	Glutamate (0.01M)	60	0.161	26	- 128	- 135	161	192
	Glutamate (0.01M), glucose	60	0.165	28	- 117	- 122	170	195
	Glucose	60	0.163	8	+ 37	+ 12	49	9
	Citrate, NH_4Cl (0.005M)	60	0.165	20	+ 195	+ 182	121	49
	NH_4Cl (0.005M)	60	0.164	7	+ 42	+ 16	43	11
	None	0	0.206	11	—	—	—	—
Chorion, guinea pig	Glutamate (0.01M)	40	0.223	81	- 93	- 23	360	193
	Glutamate (0.01M), glucose	40	0.207	76	- 85	- 19	367	195
Chorion guinea pig	None	0	0.317	29	—	—	—	—
	None	40	0.333	21	+ 30	+ 21	62	8
	Glutamate (0.005M)	40	0.294	64	- 73	- 39	217	90
	Glutamate (0.005M), glucose	40	0.316	74	- 62	- 17	234	93

an experiment in which citrate and ammonium chloride were substrates (Table 7). Although glutamate was rapidly synthesized within the tissue (as indicated by its appearance in the medium) the concentration of glutamate in the tissue remained low.

Four guinea-pig tissues, kidney cortex, spleen, lung and chorion, gave results similar to those obtained with brain in that accumulation of glutamate occurred against a concentration gradient. Examples are given in Table 7. In kidney and chorion the concentration reached was greater than in lung and spleen, but in none of the four tissues did the glutamate concentration reach the high levels which were found in brain cortex. The addition of glucose to the medium did not affect accumulation in the four tissues.

A common feature of all the tissues examined is the ability to maintain a glutamate level above that of the medium if the concentration in the medium is low, i.e. similar to that found in blood plasma. The tissues differ with respect to the final level to which they can concentrate glutamate. Testis, kidney medulla, liver, intestinal wall, choroid plexus and amnion appear to be 'saturated' with glutamate at the start, they cannot increase the initial concentration. Brain, kidney cortex, chorion, lung and spleen are able to accumulate glutamate above the starting level.

The foetal membranes and placenta were examined as examples of growing tissues. They came from guinea pigs at approximately the 45th day of gestation. The results obtained with these materials revealed no correlation between accumulation of glutamate in the tissue and rate of growth.

In several experiments a small but definite amount of glutamate was formed on incubation of tissue when no glutamate was added to the medium (indicated in Table 7 by the positive signs in the column 'glutamate metabolized'). Most probably this glutamate was derived from proteins and peptides by hydrolysis.

Table 8 *Swelling of guinea pig tissues in isotonic saline under aerobic and anaerobic conditions*

(Phosphate saline, 40 min incubation, 0.02M glucose)

Tissue	Change in wt (% of initial wt)	
	O ₂	N ₂
Brain cortex	+26	+52
Kidney cortex	0	+52
Liver	+14	+66
Lung	+1	+12
Spleen	-17	+4

Note on swelling of tissues in isotonic saline media. Reference has already been made in the Experimental section to the swelling of the tissue observed

in the present experiments. Outstanding among the observations recorded was the fact that the weight increase found after anaerobic incubation was consistently greater than that after aerobic incubation (Table 8). As the amount of medium adherent to the incubated slices must have been approximately the same for both conditions, the differences in weight increase can only have been due to different degrees of swelling. Evidently respiration is an important factor in controlling the fluid uptake of the tissues.

DISCUSSION

Tissue slices as material for the study of permeability and storage. The experiments reported in this paper show that a mechanism is operative in slices of brain cortex and other tissues which transports glutamic acid from the medium into the tissue against a concentration gradient. In guinea-pig brain cortex the transfer stopped when the difference between the internal and external concentrations was about 0.02M. Within certain limits this difference was independent of the absolute level of the external concentration. Tissue slices thus afford a system in which the mechanisms responsible for the permeability of the tissue and for the active storage of substances within the cell can be investigated under controlled conditions.

Sources of energy. The accumulation of glutamate against a concentration gradient is necessarily dependent on a source of energy. This accounts for the fact that substrates which can yield energy must be present if accumulation is to take place, and that inhibitors which interfere with the production of energy, such as sodium fluoride, also interfere with the accumulation of glutamate. Of the various substrates of oxidation, glucose was the most effective in brain, which is remarkable because lactate and pyruvate usually give rather higher Q_{O_2} values than glucose (see Elliott, Greig & Benoy, 1937). Glucose was less effective anaerobically than aerobically, it maintained the initial level but failed to support accumulation. The observation that adenosinetriphosphate had no effect should not be taken as evidence against the participation of energy-rich phosphate bonds in the energy transfer.

Energy requirements of brain. Brain, without doing external work, requires a continuous supply of energy, and it has been said that this energy is required to maintain a thermodynamically unstable structure. Few examples have been offered defining precisely in which respect the 'structure' of the tissue is 'unstable'. A high concentration of a substance in cells whose walls are permeable to the substance may be looked upon as a sign of an unstable structure. Glutamate in a concentration much above that of the blood plasma is in this sense a component of the 'structure' of brain which the forces of diffusion

tend to disintegrate. Energy must therefore continuously be added to maintain the 'structure'.

Permeability and metabolism. The present experiments provide a new demonstration of the influence of metabolic activities on cell permeability. As long as brain tissue is supplied with glucose, and the enzyme systems concerned with the energy supply from the glucose are intact, glutamate appears to permeate solely from the medium into the tissue and not in the reverse direction. But if the production of energy stops glutamate migrates from tissue to medium. Analogous observations have been recorded by Harris (1941), Wilbrandt (1940), and Maizels (1948), who studied the permeability of red blood cells for inorganic ions. Normal red cells seem to be impermeable to cations but when the rate of the energy-giving reactions of erythrocytes is lowered, either by chilling, or by inhibitors such as fluoride, or by lack of glucose, potassium and sodium diffuse through the cell wall according to the diffusion gradient. Restoration of the energy-giving reactions leads to redistribution of the cations towards their physiological state.

These observations, as well as those on swelling, make it clear that in the cases studied the dominating factor in cell permeability is not any inherent and constant physical property of the cell wall, like porosity or a lipid-sieve structure, but a mechanism dependent on the supply of energy. When this mechanism breaks down permeability changes and purely physical properties of the system come to the fore. It has of course always been appreciated that the supply of energy is an essential factor when substances are transported through cell walls in the processes of secretion and absorption. Hill (1931) and Krogh (1946) have already discussed the extension of this concept to the permeability of body cells in general, in particular also to the apparent absence of transport through the cell wall of cell constituents.

It would be premature to speculate on the nature of the mechanism by which energy is used to maintain concentration gradients. An experimental approach to the problem might be based on the hypothesis that electrical energy is the immediate form of energy which controls the movement of ions.

Accumulation of metabolites in tissues as a source of error in quantitative experiments. It has been customary to measure rates of metabolism in tissue slices by estimating the quantities of the metabolites removed from the medium. It is evident that errors arise if the substances removed are not metabolized but stored in the tissue, or conversely, if the tissue releases substances into the medium from accumulated stores. The measurement of the true metabolic rates requires in such cases the inclusion of the tissue in the analysis.

Accumulation of glutamate in micro organisms. Reference should be made to the work of Gale on Gram-positive bacteria and of Taylor on yeast (reviewed by Gale, 1948) concerning the relations between extracellular and intracellular concentrations of glutamate and other amino-acids. The phenomenon of amino-acid accumulation is in general very similar in certain micro-organisms and in animal tissues, but details such as the quantitative relations between internal and external concentration, the effects of inhibitors and of substrates show many differences. This is hardly surprising as the micro organisms themselves show great variations from species to species and within the same species with varying ages of the culture.

SUMMARY

1 Slices of guinea-pig brain cortex, when suspended aerobically in a saline medium containing glucose and L-glutamate, accumulate L-glutamate within the tissue. The transport of glutamate into the tissue occurs against a concentration gradient. Conditions for studying this phenomenon have been elaborated.

2 Transport of L-glutamate into the tissue stopped when the difference between the concentrations in the tissue and in the medium was about 0.02M.

3 The concentration of L-glutamate in the tissue fell when glucose was omitted from the medium. Substrates which supported accumulation of glutamate were glucose, fructose, L-lactate and pyruvate, but in most experiments glucose was more effective than the other substrates. Succinate, α -glycerophosphate or excess of glutamate did not prevent the fall of the initial glutamate concentration in the brain, although these substances are readily oxidized by this tissue.

4 Whilst brain slices retained and accumulated L-glutamate, the very much larger molecules of the glutamic-aspartic transaminase readily diffused from the slices into the medium.

5 L-Glutamine was taken up by brain tissue a little more rapidly than L-glutamate, but the final concentrations reached were about the same.

6 Anaerobically no appreciable accumulation occurred, but glucose delayed the fall of the initial concentration of glutamate in the tissue.

7 D-Glutamate was converted into L-glutamate by brain slices.

8 Adenosinetriphosphate abolished the inhibition of brain glycolysis by glutamate. It is suggested that the glutamate inhibition is due to an interaction between glutamate and adenosinetriphosphate to form glutamyl- γ -phosphate and that the removal of adenosinetriphosphate by this reaction is responsible for the slowing down of glycolysis.

9 Accumulation of glutamate also occurred in isolated kidney cortex, spleen, lung and chorion. Six other tissues examined maintained a concentration gradient between tissue and medium, but did not increase the glutamate concentration above the original level.

10 It is pointed out that tissue slices afford a system in which problems of permeability and storage can be studied under controlled conditions. The permeability of brain tissue for glutamate is controlled by energy-giving metabolic processes.

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Displacement Chromatography on Synthetic Ion-exchange Resins

1 SEPARATION OF ORGANIC BASES AND AMINO-ACIDS USING CATION-EXCHANGE RESINS

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The work described in Parts 1 and 2 of this communication represents an attempt to apply the principles of the flowing chromatogram to the separation of organic electrolytes on synthetic ion-exchange resins. The intention has been to develop the method for application to biochemical preparations on a fairly large scale, since the special properties of the resins render them particularly suitable for this purpose. The possibility of using an adaptation of the method for the quantitative analysis of mixtures of bases has not been considered here.

A preliminary account of this work was published in 1947 (Bendall, Partridge & Westall, 1947, Partridge, Westall & Bendall, 1947), and soon afterwards an analytical procedure based on similar principles was published by Drake (1947). Both methods are based upon the techniques described by Tiselius (1943) and Claesson (1946), who applied the principle of 'displacement development' to separations on adsorption columns of carbon or alumina

and developed a quantitative analytical technique suitable for application to small quantities of material.

The advent of the sulphonated cation exchange resins has made it possible, for the first time, to effect the quantitative adsorption of cations from solutions of neutral salts, whilst the development of ion exchangers of the polyamine formaldehyde type has allowed the removal from the resulting solution of the anions liberated in the form of free acids. Many applications of the newer synthetic resins for the purpose of removing undesired electrolytes from biological solutions have been reported (cf Platt & Glock, 1943, Partridge, 1948), but the technique to be described here seeks to take the use of ion-exchange resins a stage further by effecting the chromatographic fractionation of both the acids and the bases present in the solution with a view to the eventual isolation in a pure condition of such components as may be of interest.

In order to make the principles of the technique clear from the outset, the essentials of the method (as particularly applied to the fractionation of a mixture of bases) may be stated briefly as follows. A solution of the mixture of bases to be separated is introduced on to a column containing a cation-exchange reagent, and the components of the mixture are displaced down the column by application of a solution of a base that is more strongly

visible, the separations that take place on the column must be followed by measuring changes in composition of the effluent, and means are required whereby qualitative and quantitative information may be obtained continuously as the solution flows from the column. Various methods of following changes in the physical properties of the effluent in flowing chromatograms have been suggested (Claesson, 1946), but the three methods found most suitable for the purpose of the present work are (a) continuous measurement of electrical conductivity, (b) continuous measure-

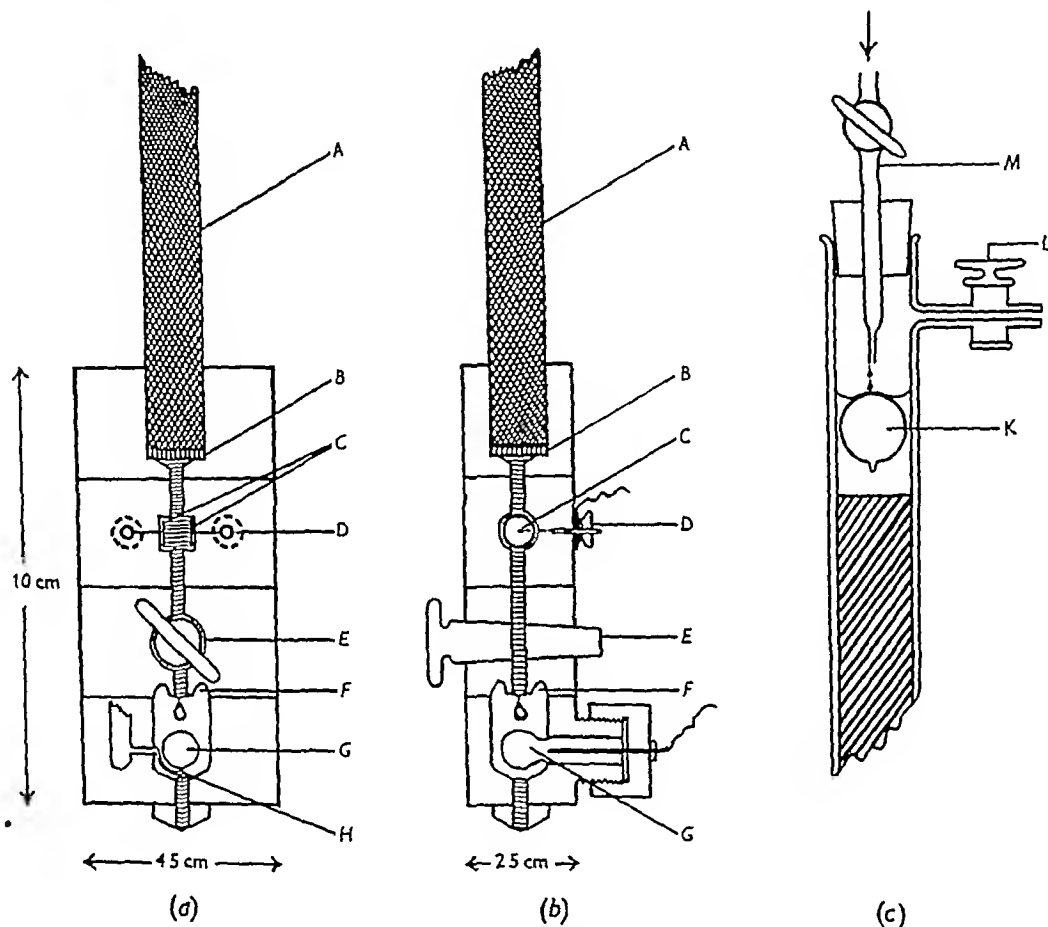


Fig 1 Column assembly (a) Front elevation, (b) side elevation, (c) upper portion of column showing inlet and float

adsorbed than any of the bases in the mixture. Once a flowing equilibrium has been established, the less strongly adsorbed bases are successively displaced by those having a stronger affinity for the ion exchanger, and the components of the mixture pass down the column in a series of discrete bands, finally flowing from it in an order dependent upon the strength with which they are adsorbed.

In the experiments described in Part 1 of this series the cation exchanger 'Zeo-Karb 215' was used. This resin may be obtained from the Permutit Co Ltd, London.

METHODS

Since in general the bands formed by electrolytes adsorbed on columns packed with ion exchange resins are not clearly

ment of hydrogen ion concentration, (c) titration of small successive volumes of effluent.

To these three physical methods may be added a fourth that depends specifically on the chemical constitution of the solute. In the separation of mixtures of amino acids it is usually of advantage to take a large number of samples from the effluent at fixed intervals, and to carry out the qualitative analysis of each sample by use of the filter paper chromatogram of Consden, Gordon & Martin (1944).

Measurement of pH and electrical conductivity. Fig 1 illustrates the form of apparatus adopted for small scale laboratory investigations. The units of the assembly were interchangeable, and consisted of blocks of 'Perspex' which were accurately machined and fitted one above the other in a frame constructed from the same material. The assembly comprised (1) a series of interchangeable blocks designed to carry columns of varying diameter, (2) a block carrying the

conductivity cell, (3) a block carrying a tap for regulating the rate of flow of the solution, (4) a block carrying the glass electrode cell. In use the blocks were sealed together by means of a thin film of soft petroleum jelly.

The column (A) was of glass and was closed by a perforated disk of Perspex (B) above which was a thin uniform layer of glass wool. The black platinum electrodes (C) were 1 cm in diameter, and were accurately fitted into the flat faces of a cylindrical cavity so that only one face of each electrode was exposed to the solution. The electrodes were connected by platinum wire to the terminals (D) mounted on the back of the Perspex block. Regulation of the rate of flow of the solution was carried out by means of a tapered tap (E) made from Perspex and lubricated with vaseline. The glass electrode (G) consisted of a bulb blown on the end of a tube of soft glass (Corning, 015) and was mounted in a cylindrical cavity (diameter 15 mm) in the lower block. The tube carrying the bulb (which in use was filled with 0.1N-HCl) was sealed at the open end by means of a threaded Perspex collar provided with a screw cap and a rubber gasket through which a silver wire passed to the terminal. The KCl bridge connexion was made by leading a fine glass capillary (H) to a position immediately below the bulb.

To avoid the possibility of electrical leakage from the conductivity cell to the glass electrode cell the liquid level in the latter was not allowed to rise above the centre of the bulb, and an annular recess was provided in the base of the tap section (shown at F in Fig. 1) in order to induce the liquid to fall on the electrode bulb in the form of small drops.

The resistance of the conductivity cell was measured by means of a 'Mullard' bridge energized by a 1000 cycle oscillator, while pH was recorded as millivolt (mV) readings by means of a 'Unicam' potentiometer.

Determination of break-through volumes When a solution of a base is passed through a cation exchange column a 'band' forms on the upper part of the column and the effluent at first consists of pure solvent only. The region of partial saturation in front of the band is called the 'boundary' of the band, and under varying conditions the boundary may be sharp or more or less diffuse. As further solution is applied the band extends downwards, and when the boundary reaches the bottom of the column the concentration of the solute in the effluent rapidly increases until finally the solution flowing out has the same concentration as that flowing in. At this point the whole of the column material is in equilibrium with the inflowing solution.

The changes in concentration of the effluent may usually be measured by direct titration, and Fig. 2 shows a volume concentration curve obtained in this way. The volume V_i (Fig. 2) represents the volume of water contained in the apparatus before the experiment begins and includes the water enclosed within and between the particles of resin. To obtain a value for V_i , the apparatus is first weighed empty. The column is then packed, and the water level adjusted to a fixed mark on the column after which the apparatus is reweighed. The weight of the dry resin is then subtracted from the difference in weights so obtained, thus giving the total weight of water initially present in the apparatus. V_0 (Fig. 2) is the volume of effluent measured to the point at which the solute first breaks through, while V_{90} is the volume of effluent measured to the point at which the outflowing solution reaches 90% of the concentration of the solution supplied to the column. V_e represents the break-

through volume calculated on the assumption that the boundary is perfectly sharp, and is derived by fixing the position of the line DB such that the area ABC equals the area ADE. Thus the weight of solute adsorbed by the resin is given by $C \times V_e$, where C is the concentration of the inflowing solute. Unless otherwise stated, the values given for V_0 , V_{90} and V_e in this paper are corrected for the small volume V_i .

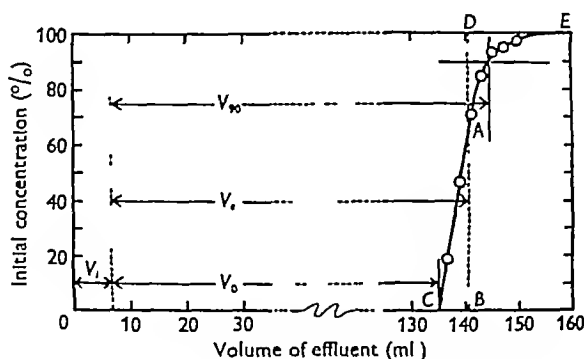


Fig. 2 Volume concentration curve for effluent from 0.042N NH_3 solution on 'Zeo Karb 215' (145 g mesh size 120-150) contained in a column 8.5 mm in diameter (volume axis shortened)

Construction and packing of the columns Faults in the construction or packing of columns may often be recognized from the shape of volume concentration curves of the type given in Fig. 2, which shows the typical curve obtained from a well packed column. With faulty columns $V_{90} - V_0$ is increased and the curve may be markedly sigmoid in shape or may show the presence of a 'foot' due to distortion of the boundary as it progresses down the column.

Various methods of packing the column were investigated. The technique usual in adsorption chromatography, in which the columns are packed dry and tamped at frequent intervals, was found to be unsuitable for use with ion exchange resins, since the expansion that takes place as the resin swells in water tends to disturb the uniformity of distribution of the particles. The method finally adopted as a standard procedure for packing the cation exchange resin 'Zeo Karb 215' was as follows. The resin, after grinding in a dry condition in a hammer mill, was graded by sieving. The graded resin was allowed to swell in 5N HCl overnight, and was then treated alternately two or three times with 2N HCl and 2N NaOH by sedimentation in a beaker. During this process a certain amount of fine material that gave rise to a cloudy suspension in water was rejected, and the final supernatant fluid was clear and colourless. The resin was then washed with distilled water until substantially free from HCl and dried at room temperature on open trays. A water-content determination was made on the sample of resin by drying to constant weight over P_2O_5 *in vacuo* at 80° , and the value was used to calculate the true dry weight of the resin introduced into the column. A weighed quantity of the resin was allowed to swell in water and was completely transferred in small portions to the column, which was previously filled with water. As the particles of resin fell to the bottom of the column, water was allowed to drip from the lower tap in order to avoid the disturbance caused by the upward displacement of water by the falling resin. In this way the column was built up as a series of flat

laminae and no attempt was made to consolidate it by tamping or tapping. The column, once made, was not allowed to drain dry and was always kept with a small depth of water above the resin. Before use the column was treated two or three times alternately with 2N HCl and N-NH₃, and after finally activating with 2N-HCl, was washed with distilled water until the conductivity of the effluent reached a steady value.

The design of the column has a marked influence on the sharpness of the boundaries obtained in separation experiments, and particular attention should be paid to the construction of the lower orifice. The type of construction shown in Fig 1 (a and b), which embodies a perforated plate (B), has given good results in practice, and was designed to ensure that the boundary leaves the column in a uniform manner and is not impeded or distorted by a dead space at the shoulders of the outlet tube.

The arrangement of the inflow to the column is also important. The liquid entering the column should in no case be allowed to flow down the side since this usually gives rise to a sloping boundary which, once it is formed, frequently persists throughout the experiment. Fig 1 (c) shows an arrangement that has proved satisfactory. The water level is maintained a little above the top of the resin and a small spherical glass float (K) is introduced in order to break the force of the falling drops of solution. The solution is introduced from a separating funnel through a glass tube (M) which is drawn out to a capillary to reduce the size of the drops. The water level in the column is maintained at a fixed position throughout the experiment by manipulation of the side tap (L).

RESULTS

Construction of 'retention isotherms' The retention of bases and ampholytes on the cation exchange column may be expressed quantitatively in the form of an 'isotherm' in which the amount of substance adsorbed/g of adsorbent is plotted against the concentration of the solution. Such curves may be plotted from measurements of V_e at various concentrations of the solute, using columns containing a known weight of resin. If ϵ is the amount of solute adsorbed (mmol/g dry wt of resin), W the dry weight of the resin, and c the concentration (mmol/ml) of the solution flowing into the column, then

$$\epsilon = \frac{V_e \times c}{W} \quad (1)$$

Fig 3 shows the retention curves obtained by this method for ammonia and a number of amino acids using 'Zeo-Karb 215'.

The curves given by the stronger bases show considerable deviation from the Langmuir adsorption equation (Langmuir, 1918). This behaviour is to be expected since in experiments of the kind illustrated in Fig 3, in which decreasing concentrations of a base are applied to the column, there are two effective variables, the concentration of the cation and the concentration of hydrogen ions. In addition, since the resin used is of the sulphonated phenol-formaldehyde type, it possesses weakly acid phenolic

hydroxyl groupings which accept cations from the more strongly alkaline solutions but are inert under conditions of lower pH.

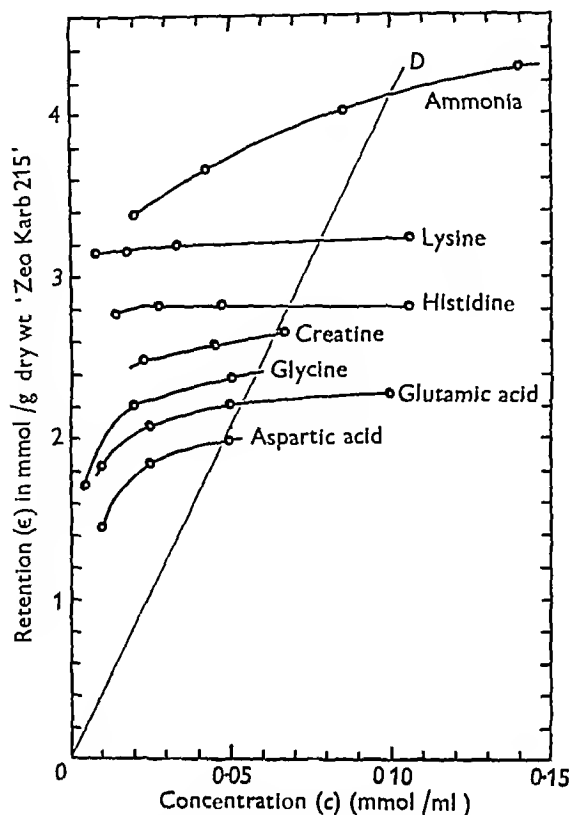


Fig 3 The retention of ammonia and a number of amino acids on 'Zeo Karb 215' (80-100 mesh). The experiments were carried out with a column 8.5 mm in diameter containing 1.44 g 'Zeo Karb' (dry wt).

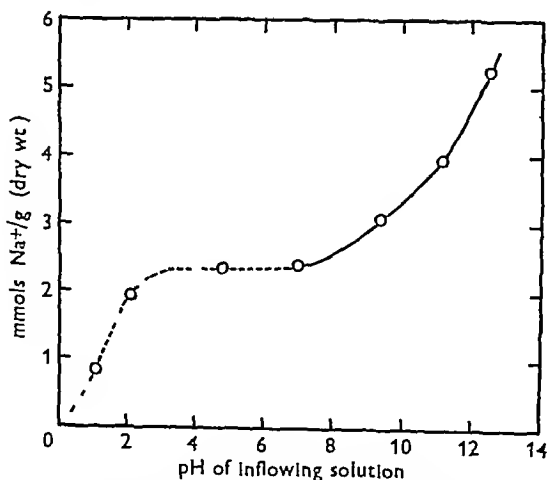
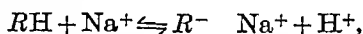


Fig 4 Adsorption of Na⁺ from a range of buffers containing NaOH (0.053N) adjusted to pH 12.1-14 by the addition of acids.

The effect of the hydrogen-ion concentration of the inflowing solution is illustrated in Fig 4 in which ϵ is plotted for the adsorption of sodium ions from a range of buffer solutions each containing

NaOH (0.053N) The solutions were adjusted to pH 12.5–11 by the addition of various acids. The buffer solutions were as follows: pH 12.5–9.35, NaOH + boric acid; pH 6.9, NaOH + NaH_2PO_4 ; pH 4.7, NaOH + acetic acid; pH 2.1–1.1, NaOH + HCl. The resulting curve of ϵ values (Fig. 4) is plainly a titration curve of the resin, but differs from the usual form of such curves in that the concentration of sodium ions in the system has been maintained at a constant value. The shape of the curve shows that there are two main absorbing groups: a strongly acid group (approx. $\text{pK} \approx 1.5$) which is presumably the sulphonic acid radical, and a series of weakly acid groups of $\text{pK} > 10$ which may be identified with phenolic residues. These latter may be modified by the formation of quinonoid structures, and for that reason may be more strongly acid in reaction than is the case with simple phenols.

In Fig. 4 the range pH 0–3 represents the region over which the sulphonic acid radicals of the resin are being titrated by the base and the reaction may be represented by



where RH is the hydrogen form of the resin. (The symbol $\text{R}^- \text{Na}^+$ is used to represent the dissociated resin salt in which movement of the cation Na^+ is restricted by the effect of the negative charges on the resin.) From this

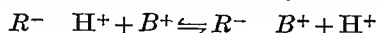
$$K = \frac{[\text{R}^- \text{Na}^+][\text{H}^+]}{[\text{RH}][\text{Na}^+]} \quad (ii)$$

The term $\frac{[\text{R}^- \text{Na}^+]}{[\text{RH}]}$ may be replaced by $\frac{\epsilon}{(\epsilon_{\text{sat.}} - \epsilon)}$, where $\epsilon_{\text{sat.}}$ is the maximum adsorption of Na^+ in mmol/g of resin (dry wt.) by the sulphonic acid residues of the resin. Thus

$$K = \frac{\epsilon}{(\epsilon_{\text{sat.}} - \epsilon)} \frac{[\text{H}^+]}{[\text{Na}^+]} \quad (iii)$$

A value for $\epsilon_{\text{sat.}}$ is given by the ordinate of the horizontal section (pH 5–6) of the experimental curve (Fig. 4), and K may be calculated by use of equation (iii) from any experimental point on the curve in the range pH 0–3. The dotted portion of the curve in Fig. 4 (between pH 0 and 7) was calculated from (iii) taking $\epsilon_{\text{sat.}} = 2.35$ and $K = 0.82$.

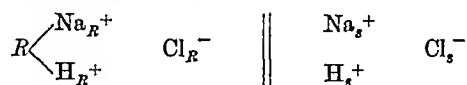
Over the region pH 3–7, ϵ approaches $\epsilon_{\text{sat.}}$ and the system becomes insensitive to changes in $[\text{H}^+]$. In this condition the resin acid may be regarded as fully ionized with respect to the sulphonic acid groups and the reaction with a cation B^+ may be written



In this system the negatively charged centres R^- are restricted in movement by attachment to the polymer, and a condition approaching electrical neutrality is maintained by the orientation of cations, hydrogen ions or the ions of ampholytes

about the negative centres. The adsorption of a cation B^+ will therefore be affected by conditions relating to the size and charge of the cation and its activity in the external solution. Where anions are present in the external solution they will also be present to some extent in the resin gel and 'adsorption' of anions by the resin may be considerable where B^+ is polyvalent.

By application of the Donnan equation to the equilibrium between the resin gel and the external solution in the system



Bauman & Eichhorn (1947) derive the relation

$$\frac{[\text{Na}_R]}{[\text{H}_R]} \frac{[\text{H}_s]}{[\text{Na}_s]} = k = \frac{f\text{H}_R}{f\text{Na}_R}, \quad (iv)$$

where $[\text{Na}_R]$ and $[\text{Na}_s]$ are the concentrations of Na^+ in the resin phase and the solution phase, respectively, and $[\text{H}_R]$, $[\text{H}_s]$ are the corresponding concentrations of H^+ . $f\text{H}_R$ and $f\text{Na}_R$ are the molar activity coefficients of H^+ and R^+ in the resin phase. Equation (iv) was shown to be valid for dilute solutions of salts of monovalent inorganic bases. From their experimental results Bauman & Eichhorn (1947) were led to the opinion that the ion exchange equilibria between monovalent metallic cations on Dowex 50 (a resin that contains $-\text{SO}_3\text{H}$ as the only acidic radical) is determined simply by the differences in activity coefficients between the outside solution and the very highly concentrated solutions in the resin gel, without involving any specific affinity of $-\text{SO}_3\text{H}$ groups for one ion over another.

The exchange reaction taking place between two weak organic bases is, however, significantly different from that occurring between the salts of alkali metals, since in the former case one base may depress the dissociation of the other, and the cations of the stronger base will therefore be more available to satisfy the negatively charged sites on the resin. Thus in the special case of the exchange of organic cations between the resin and a solution of a weak base or amino-acid, the effect of the stronger base in suppressing the ionization of the weaker base in the liquid phase becomes an important consideration, and, as will be shown later, there is a strong presumption that this effect is a factor in determining the order of displacement of a series of organic bases and ampholytes.

The equation of Bauman & Eichhorn (iv) is of the same form as the mass action equation (iii) and may be reduced to

$$k = \frac{\epsilon}{\epsilon_{\text{sat.}} - \epsilon} \times \frac{[\text{H}_s]}{[\text{B}_s]}, \quad (v)$$

where the two constants k and $\epsilon_{\text{sat.}}$ refer specifically to the exchange of hydrogen ions for the cation B^+ .

If the pH of the equilibrium solution is maintained at a constant value, $[H_+]$ may be neglected and equation (v) becomes

$$h' \epsilon_{\text{sat}} - h' \epsilon = \frac{\epsilon}{[B_+]} \quad (vi)$$

This equation is of the same form as the 'adsorption isotherm' of Langmuir (1918), and describes the adsorption of cations by the resin acid from a solution of a base at constant pH. It should therefore be applicable to the adsorption of the acidic and neutral amino-acids since solutions of these show little pH change on dilution. The validity of equation (vi) for the adsorption of the amino acids may be demonstrated by plotting the values of ϵ against $\epsilon/[B_+]$. From the data in Fig 3 the plots for glutamic acid, aspartic acid and glycine fall on a series of straight lines at concentrations within the useful range.

The separation of mixtures of bases and ampholytes
Fig 5 gives the results of a typical experiment in which a mixture of three amino acids was separated by displacement with ammonia solution. In this experiment a mixture of aspartic acid, glycine and histidine was chosen for the reason that the isoelectric points of these amino acids are well separated on the pH scale, and thus they show no tendency to form mixed bands on the column.

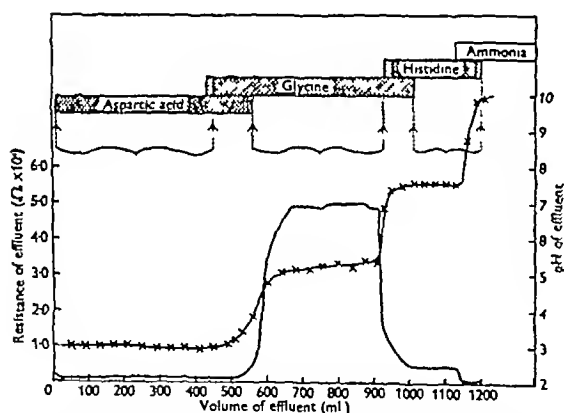


Fig 5 The separation of aspartic acid, glycine and histidine by displacement with 0.10N NH_3 . Smooth curve, resistance, x—x, pH values

The column used was 15 mm in diameter and 400 mm high. It contained 28 g (dry wt) of 'Zeo-Karb 215' which had previously been ground to 40–60 mesh/in and prepared according to the directions already given. An aqueous solution (1 l) containing L-aspartic acid, 2.0 g, glycine, 2.0 g and L-histidine monohydrochloride, 2.7 g was passed through the column at a rate of 250 ml/hr, after which the column was washed with a further 500 ml distilled water in order to ensure the removal of residual hydrochloric acid from the amino acid hydrochloride.

Displacement was carried out with 0.10N- NH_3 (1210 ml), which was introduced at a rate of 200 ml/hr, and the effluent was collected in 10 ml fractions. The conductivity of the effluent was recorded continuously during the run, and the results are plotted in the form of a smooth curve in Fig 5. The figure also shows pH readings determined on every fourth fraction after the run was complete.

It will be seen from the form of the curves that the course of the fractionation can be followed from the pH and conductivity measurements alone, but in order to provide a more sensitive check on the purity of the fractions, a drop of the solution was taken from each 10 ml fraction and introduced on to a filter paper chromatogram. This was irrigated with phenol and developed with ninhydrin according to the technique of Consden *et al* (1944). The resulting chromatogram is represented diagrammatically in Fig 5. The lightly hatched areas at the leading and trailing edges of the amino-acid bands represent the presence of the amino-acid as a trace only. It is clear from the diagram that the effluent collected in fractions 1–42 contained aspartic acid only, while those in fractions 56–92 and fractions 101–114 contained pure glycine and pure histidine respectively. Fractions 115–121 contained histidine and ammonia, but since the latter may readily be removed by evaporation under reduced pressure, the material was suitable for addition to the main histidine fraction.

Fractions 1–44, 56–92 and 101–121 were combined, and in each case the solution was evaporated to small bulk under reduced pressure. The concentrated solutions were then evaporated to dryness under vacuum. A colourless crystalline product was obtained in each case, in the yields given below, the purity of the fractions was confirmed by analysis for nitrogen.

	Yield (g)	Yield (%)	N (%)	
			(Found)	(Calc)
Aspartic acid	1.63	81.5	10.5	10.5
Glycine	1.54	77.0	18.7	18.7
Histidine	1.77	80.5	27.0	27.1

The concentration of the solutes in the effluent solutions
For the purpose of designing a suitable fractionation procedure it may be necessary to calculate the concentration at which each component will be delivered from the column under given conditions. The graphical method of Claesson (1946) may be adopted for this purpose. In Fig 3 a line OD is drawn from the origin (O) to a point D on the isotherm of the displacement developer which represents the concentration of the developer to be applied. The point at which the line OD cuts the isotherm of any component (A) gives the concentration at which the solution of A will flow from the column.

The gradient of $0D$ is $\Delta\epsilon/\Delta c$ and is a function of the break-through volume of the developer, thus

$$\frac{W\Delta\epsilon}{\Delta c} = \frac{W\epsilon_d}{C_d} = V_d, \quad (\text{vi})$$

where W is the weight of resin in the column and ϵ_d , C_d and V_d refer to the developer D . Similarly, for any other component A ,

$$\frac{W\Delta\epsilon}{\Delta c} = \frac{W\epsilon_a}{C_a} = V_a, \quad (\text{vii})$$

where ϵ_a , C_a and V_a refer to the point of intersection of the line $0D$ with the isotherm of A . Thus the point of intersection determines the concentration of the component A such that $V_a = V_d$. From this it is clear that if the component A is displaced by component D (applied at concentration C_d) and the column is sufficiently long for the establishment of a flowing equilibrium, then A will flow from the column at the concentration C_a . If A is initially applied to the column at a concentration lower than C_a , then the band due to A contracts on application of the developer until the adsorbed solute reaches equilibrium with a solution of concentration C_a , similarly, if A is initially adsorbed from a concentration higher than C_a the band increases in width during development.

The argument given above is valid for a displacement experiment only in so far as the component is in the same ionic environment as prevails during the determination of the individual isotherms. This condition is most probably completely fulfilled where weak electrolytes are adsorbed from organic solvents by such reagents as carbon, but may not be fulfilled in practical experiments with ion exchangers. Nevertheless, where free bases such as ammonia, sodium hydroxide or barium hydroxide were used as displacement developers, the concentrations found by experiment showed good agreement with the calculated values. Displacement with solutions of salts, however, introduces the complication of an anion, and under such circumstances a calculation based upon the isotherms determined for the free bases is rendered valueless.

Where displacement experiments are carried out on a mixture of solutes it is important to ensure that the concentration at which the least soluble component is displaced does not greatly exceed its solubility in the solvent, for should this occur anomalous results may be obtained due to the formation of crystals in the column. However, in practice, it is often possible to displace a solute at a concentration slightly in excess of its solubility, and in this case crystals of the pure solute may appear in the fractions taken from the effluent. This effect was observed in the separation experiment illustrated in Fig 5. In this experiment 0.10N-ammonia was used for displacement, and crystals of aspartic acid

appeared in the effluent solution soon after the fractions were collected.

The order of displacement In displacement chromatograms carried out on adsorbents such as carbon, the order of displacement of a series of solutes is given directly by the relevant isotherms, the solutes that are adsorbed to a greater extent displacing those adsorbed less strongly (Claesson, 1946). Where ion exchangers are used this rule is not universally applicable, and anomalies are noted particularly where mixtures of monovalent bases, divalent bases and amino-acids are displaced from cation exchange columns. Table 1 lists a number of bases and amino acids in the order in which they are displaced from 'Zeo-Karb 215' and shows that this order does not follow exactly the order of decreasing values of ϵ . The brackets in the table include pairs of solutes that form mixed bands on the column.

If we consider a point on a column at the boundary between two bands, both solutes will be present in the liquid phase, and the stronger base will tend to suppress the ionization of the weaker. Thus the stronger base will be preferentially adsorbed, and for this reason it would be expected that the pH of the emerging solutions would give a more exact guide to the order of displacement than the values of ϵ , which are affected by the specific effects of the cations. The results given in Table 1 bear out this

Table 1 Retention data for a number of bases and ampholytes on 'Zeo-Karb 215'

(The bases are given in the order of displacement, but the substances included in the brackets do not separate on the column. ϵ is the amount of solute (mmol.) retained by 1 g of dry resin from a solution of concentration c (mmol./ml) when this is passed continuously through a column of the resin.)

Substance	Equilibrium conc (c) (mmol./ml)	ϵ (mmol/g dry wt)	pH of emerging solution
{NaOH	0.071	4.77	12.9
{Ba(OH) ₂	0.05	3.38	12.7
Ammonia	0.05	3.70	11.0
Lysine	0.05	3.20	9.7
Anserine	0.035	2.35	8.3
{Carnosine	0.035	2.35	8.2
{Creatinine	0.041	2.75	8.2
Creatine	0.05	2.60	7.0
Glycine	0.05	2.36	6.0
{Serine	0.05	2.26	5.1
{Glutamic acid	0.05	2.20	3.2
Aspartic acid	0.05	1.98	2.8

general rule, thus, although in general the values of ϵ and the pH of the emerging solutions run parallel, there are certain irregularities, and the order of displacement appears to be more accurately given by the order of pH. Subsequent experience has shown that the pH rule is useful for the purpose of predicting the expected position of bases and amino acids in flowing chromatograms. The pH of a solu-

tion of an amino-acid, as it flows from a cation-exchange column, has near its isoelectric point, provided that the mixture of solutes is displaced by a base and anions are excluded from the system

The behaviour of serine presents an important exception to the pH rule since this amino-acid forms a mixed band with glutamic acid

Factors influencing the width of the boundaries
Fig 2 represents the variation in concentration of the solute as a band emerges from the column. The values for V_i , V_{90} , V_0 and V_e are readily determined experimentally, and it is clear that the expression $V_{90} - V_0$ gives an indication of the sharpness of the front leaving the column. The height (λ_{90}) of the cylinder of column material occupied by the boundary of the front is given by

$$\lambda_{90} = \frac{(V_{90} - V_0)}{V_e} L, \quad (1x)$$

where L is the length of the column in cm. λ_{90} may be called the 'boundary width' and since $(V_{90} - V_0)/V_e$ is independent of the cross-sectional area of the column and varies inversely as its length, λ_{90} is independent of both the diameter and the length of the column. The value of λ_{90} is characteristic of the sample of resin, and is increased with increasing particle size and increasing rate of flow of solution (cf Glueckauf & Coates, 1947). Since λ_{90} is dependent upon the rate at which equilibration is attained its value may be affected by variations in temperature and should be greater with solutes of high molecular weight. Measurements of λ_{90} are most easily carried out by examining the front given by a single basic component displacing H^+ from the ion exchanger. However, the boundary between two basic components may also be examined, and in those cases where the two components do not differ greatly in strength as bases the value of λ_{90} is frequently found to be very large. This subject has been discussed in relation to the shape of the two-component isotherm by Glueckauf (1947).

In Fig 6 values of λ_{90} are given for a number of samples of 'Zeo-Karb 215' of different particle size which were graded by sieving in the dry state. The solution used for the determinations of $(V_{90} - V_0)/V_e$ was in each case 0.042N- NH_3 , and all the results were obtained with 1.44 g of resin (dry wt) packed in a column 8.5 mm in internal diameter. In the figure, λ_{90} has been plotted against the rate (S) at which the boundary travels down the column in cm/hr. S may be measured independently of the dimensions of the column, and for this reason it was used in preference to the rate of flow of the solution (v), S and v are related by the expression

$$S = L \frac{v}{V_e}, \quad (x)$$

where v is expressed in ml/hr

Fig 6 gives a clear indication of the effect of increasing particle size on the width of the boundary obtained and also shows a rather smaller effect in the same direction due to increasing the rate of flow of the solution. The value of λ_{90} is clearly related to a power of the average particle diameter and, except at small values of $(V_{90} - V_0)$ is nearly proportional to the rate of flow of the solution. This is in general agreement with the predictions of Glueckauf (1947).

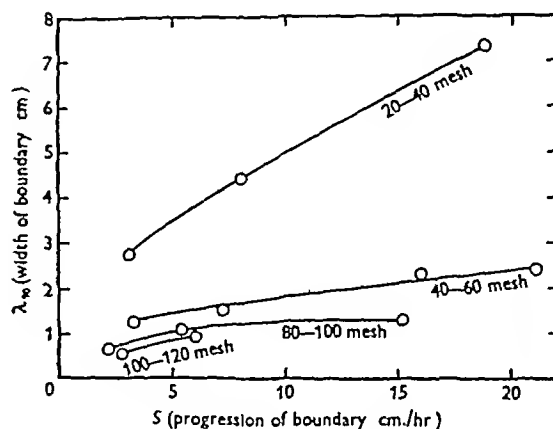


Fig 6 The effect of particle size and rate of flow on the width of the boundary of an ammonia front (NH_4^+ displacing H^+ from 'Zeo Karb 215')

The determination of values for λ_{90} for a series of sieve-graded sizes offers a method for assessing the suitability of a resin for the purpose of displacement chromatography, and experience so far obtained indicates very large differences in λ_{90} for various resins. However, in comparing the boundaries given by two different resins for the same solute, the adsorptive capacity of the resins for the solute should be taken into account, if one of the resins adsorbs less of the solute, then a longer column can be used to handle a given weight of the solute and the width of the boundary that can be tolerated will increase proportionately. For this reason it may be preferable to compare the values of $\lambda_{90} \times \epsilon$ for a series of related resins of differing adsorption capacity, rather than the direct values of λ_{90} . The performance of 'Zeo-Karb 215' was excellent when compared with that of other resins investigated, and inspection of Fig 6 shows that, with this resin, little advantage is obtained from grinding the resin to a particle size smaller than 80-100 mesh/m. For this reason 80-100 mesh powder has been adopted as standard for smaller columns, but since, for longer columns, there is advantage to be obtained from the higher rates of flow obtainable with larger particles, a powder sieving between 40-60 mesh has usually been used for experiments on a larger scale.

The values for λ_{90} given in Fig 6 were obtained for ammonia solution, but parallel experiments carried out on more basic substances than ammonia ($NaOH$,

Ba(OH)₂ and arginine) gave much broader boundaries, while the weaker bases (creatine, creatinine, glycine) gave values for λ_{90} rather lower than those given by ammonia. The volume-concentration curves for the stronger bases were very sharp at the point of initial break-through, but became very flat as the concentration of the effluent reached 50–60 % of that of the inflowing solution. This effect is clearly illustrated by Fig 7, in which the volume-concentration curves have been plotted for a series of buffer

pure material recoverable from the band of the first component (A) issuing from the column is given by

$$\text{percentage yield} = \left(1 - \frac{\lambda_{90}^b}{2l_e^a}\right) \times 100, \quad (\text{xI})$$

and that of any other component (M) is given by

$$\text{percentage yield} = \left(1 - \frac{\lambda_{90}^m + \lambda_{90}^n}{2l_e^m}\right) \times 100 \quad (\text{xII})$$

(Symbols are as indicated in Fig 8)

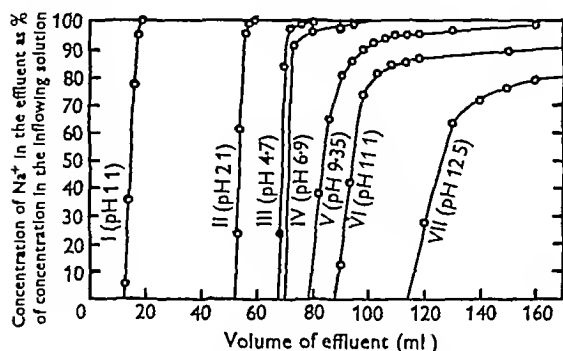


Fig 7 Volume concentration curves showing the shape of the fronts obtained on 'Zeo Karb 215' (80–100 mesh) with buffer solutions containing Na⁺ (0.053M) and various anions: I, NaCl + HCl (pH 1.1), II, NaCl + HCl (pH 2.1), III, Na acetate + acetic acid (pH 4.7), IV, NaH₂PO₄ + NaOH (pH 6.9), V, boric acid + NaOH (pH 9.35), VI, boric acid + NaOH (pH 11.1), VII, NaOH (pH 12.5)

solutions in which the concentration of the Na ion was maintained at 0.053M and the pH adjusted by addition of various anions. The experiments were carried out on 'Zeo Karb 215' (1.44 g dry wt, 80–100 mesh) packed into filtration tubes 9 mm in diameter to form a column 7.5 cm high. At low values of the pH the fronts were in every way typical of those given by weak bases, but under strongly alkaline conditions the fronts became very diffuse, although they remained sharp at the point of break-through and showed no tendency towards a sigmoid shape.

The diffuse character of the boundaries at high values of pH is believed to be due to the reaction of strong bases with the free phenolic-hydroxyl groups in the resin, and fronts of the shape found would be expected if this reaction were slower than the reaction of the bases with —SO₃H. The poor boundaries given by the stronger bases, together with the excessive swelling of the resin that occurs in strongly alkaline solutions, renders 'Zeo-Karb 215' rather unsuitable for use with bases stronger than ammonia. This is unfortunate, since the important amino-acid arginine is included in this category.

The relation of λ_{90} to the yield of pure component obtainable from a separation. Inspection of Fig 8 shows that the approximate percentage yield of

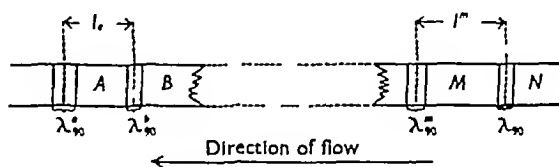


Fig 8 Diagram of the bands (A, B, M, N) on a displacement chromatogram showing the boundaries of the bands (boundary width, λ_{90}^a , λ_{90}^b , λ_{90}^m , λ_{90}^n)

In some cases (where the values for λ_{90} are small, and the components in the system are well separated in strength as bases) it may be sufficient to make the simplifying assumption that all the values for λ_{90} are equal, in which case the percentage yield of the first component becomes

$$\left(1 - \frac{\lambda_{90}}{2l_e^a}\right) \times 100, \quad (\text{xIII})$$

and that of any other component (M) is

$$\left(1 - \frac{\lambda_{90}}{l_e^m}\right) \times 100 \quad (\text{xIV})$$

The degree to which this procedure is valid is indicated by the data in Table 2, which lists values for λ_{90} for a number of boundaries on 'Zeo Karb 215'

Table 2 The width (λ_{90}) of various boundaries on 'Zeo-Karb 215'

Boundary*	Mesh/in	Rate (S) (cm/hr)	λ_{90} (cm)
H ⁺ ammonia	40–60	10	2.00
H ⁺ ammonia	80–100	10	1.25
H ⁺ lysine	80–100	5	2.40
H ⁺ histidine	80–100	5	2.56
H ⁺ glycine	80–100	5	0.56
H ⁺ serine	80–100	5	1.27
H ⁺ glutamic acid	80–100	5	2.10
H ⁺ aspartic acid	80–100	5	2.10
Histidine ammonia	80–100	5	2.14
Glycine histidine	40–60	10	3.10
Creatinine ammonia	80–100	5	0.80
Glycine creatinine	80–100	5	0.80
Aspartic glycine	40–60	10	3.50

* 'H⁺ ammonia' indicates the boundary of a front due to ammonia progressing down a column of the cation exchanger in its acid form at a rate of 8 cm/hr, 'histidine ammonia' indicates the boundary of a front due to ammonia when this is displacing histidine from the cation exchanger.

The boundaries in which a base displaces H^+ are generally rather narrower than those in which one base is displaced by another. However on the 80–100 mesh resin the values lie in the range 0.56–2.56, while on 40–60 mesh resin the corresponding range is 2.00–3.50.

For the purpose of calculating the minimum length of column required to carry out a specified separation, and to form an estimate of the expected yield, we may adopt a tentative average value for λ_{90} . If this value is taken as $\lambda_{90} = 3.0$ for separations on 40–60 mesh resin at $S = 10$ cm/hr, the 'expected yields' for the separation shown in Fig. 4 may be calculated as follows.

The column contains 0.7 g (dry wt) of 'Zeo-Karb 215'/cm length. Since 0.10N- NH_3 solution was used for displacement the data in Fig. 3 shows that, at equilibrium, ϵ (histidine) = 2.82 mmol/g, ϵ (glycine) = 2.4 mmol/g and ϵ (aspartic acid) = 1.98 mmol/g. The heights of column occupied by histidine, glycine and aspartic acid are, therefore, 7.2, 15.9 and 12.8 cm respectively. Thus the 'expected yields' of aspartic acid and histidine (from (xiii)) are 88.4 and 79.2% respectively, while the expected yield of glycine (from (xiv)) is 81.2%. These figures compare with the values found: aspartic acid, 81.5%, histidine, 80.5% and glycine, 77%.

DISCUSSION

The application of the principle of displacement chromatography to columns packed with cation-exchange resins enables a complicated mixture of bases and ampholytes to be separated into a series of cuts, each of which contains those components of the mixture which have closely similar strength as bases. Thus a mixture of amino-acids may be resolved into a series of fractions each containing ampholytes of similar isoelectric point. The isolation of pure components (from a single passage through the column) may only be expected when the initial mixture does not contain bases of closely similar strength, and in general, for ampholytes, a difference in isoelectric points of 0.5–1.0 pH unit is required to effect a useful separation between any two components.

The present communication is confined to experiments with individual bases and amino-acids and to the separation of simple mixtures, the purpose of the work being to establish the principles of the method, to collect adsorption data and to acquire a detailed picture of the relevant properties of a typical commercial resin of proven suitability to the purpose. A simple theoretical treatment has been outlined, but the physics of the rather complicated processes involved has not been pursued beyond the point required to develop a working rationale.

It is inherent in the nature of the displacement chromatogram that single-component bands over-

lap with one another over a narrow solution suitably been termed the 'boundary of the' for 15 min at 37°, of the boundary determines the fraction added. The component which may be isolated in a pure phenol determinant (King, 1947) and is affected by the experimental arrangement adopted and by the properties of the resin. Two properties of the resin are therefore of overriding importance, the adsorptive properties under different conditions of hydrogen-ion concentration and the width of the boundaries observed under standard experimental conditions.

These two factors have been studied in some detail and the results obtained provide the data required for the rational planning of attempts to fractionate complicated mixtures of biological origin on a fairly large scale.

SUMMARY

1 The principle of displacement chromatography has been applied to the separation of bases and ampholytes on columns packed with the synthetic cation-exchange resin 'Zeo-Karb 215'.

2 Separations were followed by observing changes in the composition of the effluent solutions. Four general methods were adopted for this purpose: (a) continuous measurement of electrical conductivity, (b) continuous measurement of pH, (c) titration of successive small fractions, (d) qualitative analysis of successive fractions by means of the filter-paper chromatogram.

3 Data for the adsorption of various bases and amino acids on 'Zeo-Karb 215' are given in the form of retention isotherms. The curves are satisfied by the well-known equation of Langmuir, provided the change in pH with dilution of the solute is small.

4 Effective separation between two components of a mixture of ampholytes was obtained on the column, provided that the components were separated by more than 0.5–1.0 pH unit in isoelectric point.

5 The effect of hydrogen-ion concentration on the adsorption of bases by 'Zeo-Karb 215' was investigated, over the range pH 1–7 the sulphonic acid residues in the resin constituted the only reacting group. Under more alkaline conditions, phenolic residues also react with bases.

6 The reaction of phenolic residues with strong bases reaches equilibrium slowly, and for this reason resins that contain residual phenolic groups are unsuitable for the separation of bases stronger than ammonia.

7 The effect of the particle size of the resin on the width of the boundaries of single component bands was investigated. Fine grinding was found to be essential for the production of sharp bands, and for general use a resin graded between 40 and 60 mesh/in. wire sieves was satisfactory.

8 An increase in the rate of progression of the bands down the column decreases the sharpness of the bands. On 'Zeo Karb 215' (40-60 mesh/in) a rate of progression of 10-15 cm/hr gave satisfactory results.

9 Equations have been derived permitting the calculation of the proportion of the column occupied by a component, the width of the boundaries and the expected yield of pure components in separation experiments.

10 Details of a specimen separation are given, and the yields obtained compared with estimates derived from theory.

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Purification of Alkaline Phosphatase

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Thoai, Roche & Sartori (1944) claimed to have isolated and crystallized the alkaline phosphatase (phosphomonoesterase) of ox kidney. The isolation of similar crystals from dog intestinal mucosa by the same workers has been reported by Roche (1946). These authors pointed out that the crystals lost their activity after a few recrystallizations. This they attributed to a denaturation or loss of coenzyme. By dialysis and reactivation Thoai, Roche & Roger (1947) obtained amorphous preparations which were more active than the crystals. The procedure adopted for the preparation and purification of the enzyme was a modified Albers & Albers (1935) technique.

Following the same procedure, we have obtained very similar crystals which, in the first instance, showed high phosphomonoesterase activity. But after careful purification of these crystals it has been found that they are principally composed of inorganic matter which appears to be magnesium orthophosphate. After careful dialysis for a prolonged period the crystals can no longer be obtained from the dialyzed enzyme preparations by any procedure tried, but the enzyme activity is not affected and can be completely restored by adding magnesium to the buffer substrate mixture.

We have also tried other effective procedures for

the further purification of phosphatase, e.g. tryptic digestion followed by prolonged dialysis (cf Schmidt & Thannhauser, 1943, Abul-Fadl & King, 1949). The products thus obtained show much higher activity than the mixtures containing the crystals, but several attempts to crystallize such protein material, of very high phosphatase activity, have been unsuccessful.

EXPERIMENTAL

Preparation of phosphatase extracts and purification by acetone

Fresh healthy ox kidneys are decapsulated, freed from fat, well washed and twice minced in a machine. They are then thoroughly mixed with an equal weight of 25% aqueous acetone containing 10% (v/v) of toluene and ethyl acetate (1:1). Autolysis is allowed to proceed at room temperature for 2-3 days with occasional thorough shaking. The mixture is strained through muslin, filtered clear, and then treated in the cold with acetone to a concentration of 55%. The precipitate obtained is separated by centrifuging, washed twice with pure acetone and dried *in vacuo*. A 2% aqueous filtered solution of the precipitate is then subjected to fractional precipitation with cold redistilled acetone at 0°. The fraction obtained at an acetone concentration of 38-50% is mainly protein in nature, and possesses considerable phosphatase activity. When left for 24-36 hr in the cold room (at 5°) it sets into a crystalline mass. These crystals were claimed by Thoai *et al* (1947) to be the crystalline enzyme.

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Dog intestines from normal animals have also been used for similar preparations. After thorough rinsing with saline solution, the gut is cut open longitudinally, and the mucosa is scraped off with the aid of wooden spatulas. Autolysates of intestinal mucosa, prepared as described for kidneys, have yielded highly active enzyme concentrates from which similar crystals have also been obtained.

Purification by tryptic digestion and dialysis

The fractions obtained between 38–50% acetone concentration were treated with a 0.2% solution of a highly active purified trypsin obtained from Armour Ltd.

The pH was adjusted to 8.0 with Na_2CO_3 solution and the mixture incubated at 37° for 3–4 hr. The preparation was then shaken with 5% kaolin and centrifuged or filtered. This procedure was repeated twice to remove any remaining trypsin. The phosphatase activity was not affected. The enzyme was now precipitated in 0.9 saturated $(\text{NH}_4)_2\text{SO}_4$ solution. The precipitate was collected by centrifuging, and dialyzed in collodion bags at room temperature for 3–4 days, against a large volume of distilled water frequently changed. The activity and total N were determined each day, and dialysis was stopped when the activity/mg total N remained constant for two successive estimations.

Activity determination

Activity has been determined in two ways: (a) in Roche P units, by estimation of orthophosphate liberated from 0.02M β -glycerophosphate in veronal buffer at pH 9 at 37°, (b) in King phenol units, by estimation of phenol liberated from 0.005M sodium phenylphosphate in Na_2CO_3 – NaHCO_3 buffer at pH 10 at 37°. Determination of the activity by the two methods allows of comparison with former results published both by ourselves and by other authors.

Magnesium was added to give a concentration of 0.01M. Phosphorus was determined by Briggs's (1922) method or those of King (1932) and Allen (1940), phenol by the Folin Ciocalteu method (for details see King, 1947), and total N by the micro Kjeldahl procedure.

(a) *Determination of activity in terms of Roche phosphate units* In a test tube marked at 10 ml are placed 4 ml veronal buffer (pH 9), together with 2 ml 0.1M-sodium glycerophosphate and 1 ml 0.1M magnesium acetate. The volume is adjusted to 9.9 ml, with distilled water, and the tube allowed to stand in a 37° water bath for 5 min. The highly active enzyme solution (0.1 ml) is added and hydrolysis is allowed to proceed for exactly 5 min. Trichloroacetic acid (25%, 1 ml) is added to stop enzyme action and to precipitate any proteins. (Generally the highly active preparations contain negligible amounts of precipitable proteins.) The solution is filtered if necessary, and the inorganic phosphate determination carried out. Controls without any enzyme solution and blanks with enzyme added after acidification must be run parallel with the tests. If the amount of inorganic phosphate exceeds 0.3 mg P in the whole tube (corresponding to about 5% hydrolysis) the determination is repeated with a conveniently diluted enzyme solution. The Roche unit of phosphatase is defined as the amount of enzyme which will liberate 1 μg P/min.

(b) *Activity by King phenol units* The Na_2CO_3 – NaHCO_3 buffer of pH 10 (2 ml, Delory & King, 1945), 0.02M disodium phenylphosphate solution (1 ml) and 0.04M magnesium acetate (1 ml) are mixed in a test tube and warmed to 37°.

After addition of 0.2 ml of the enzyme solution suitably diluted, and incubation of the mixture for 15 min at 37°, 1.8 ml dilute (1 in 3) Folin Ciocalteu reagent are added. The solution is mixed, centrifuged if necessary, and phenol determined in 4 ml of the clear supernatant fluid (cf King, 1947). Controls and blanks are also used. The unit of phosphatase is defined as the amount of enzyme which will liberate 1 mg phenol/15 min.

RESULTS AND DISCUSSION

Dog intestinal phosphatase Table 1 shows the activity of dog intestinal preparations at different stages of purification. The activity of the mucosa autolysates has been found to vary considerably from one preparation to another. It is important to work with intestines obtained from healthy, well-fed animals, showing no sign of abnormality. Preparations from ill or starving dogs yield solutions of low activity. The results shown in Table 1 are those normally obtained from satisfactory preparations.

Table 1 *Phosphatase activity of dog intestinal preparations during different stages of purification*

Preparation	Activity	
	Phosphate (Roche units/mg total N)	Phenol (King units/mg total N)
1 Autolysis for 3 days in 25% acetone (Autolysate filtered clear, light yellow)	650–800	70–85
2 Crude Albers, precipitated by 55% acetone from above autolysate	2000–2400	240–300
3 Fractional precipitation by acetone 38–50%, 0–4°	5000–8000	600–890
4 Tryptic digestion followed by prolonged dialysis	9360–15700	1172–1600

The amount of mucosa obtained from the whole small intestine from a normal dog is generally about 100 g which is left to autolyse as described above. The clear filtered autolysate is generally light straw-yellow in colour and has a total nitrogen content of about 0.7–1.6 mg/ml. The crude Albers preparation obtained by precipitating with 55% acetone in the cold has a higher activity (units/mg total nitrogen) than the autolysate, but its comparatively low total nitrogen content (7–9%) indicates contamination with non-nitrogenous materials (possibly carbohydrates) as well as inorganic matter.

On fractional precipitation with cold pure redistilled acetone the fractions obtained below 38% acetone concentrations are relatively poor in enzyme activity. The fractions obtained with higher acetone concentrations (38–50%), on the other hand, are rich in enzyme activity. By carrying out this stage carefully in a cold room at approximately 0°, adding the chilled pure acetone gradually with constant stirring and separating every fraction by thorough

centrifuging in the cold room, it is possible to obtain highly active products, containing about 12–14 % nitrogen

This fraction, however, is always contaminated with inorganic matter, consisting mainly of magnesium phosphate which crystallizes when the preparation is kept in the cold. The residual acetone evaporates slowly, and the inorganic crystals are gradually formed in the viscous protein solution, which adheres intimately and takes the shape of the crystals, thus giving the impression that the enzyme itself has crystallized.

The enzyme preparation at this stage could not be further purified by fractional precipitation with acetone. We have tried several ways of purification involving fractional salting out with ammonium sulphate, adsorption with alumina and kaolin, simple dialysis and electrodialysis, but none has given satisfactory results.

Tryptic digestion under the described conditions, however, followed by treatment with kaolin, salting out with ammonium sulphate and then prolonged dialysis, has been found very effective in achieving further purification. The relative stability of the intestinal phosphatase towards dialysis has been an additional favourable factor in effecting purification of this enzyme after tryptic digestion in this way. The preparation obtained after dialysis is an almost colourless, highly active, aqueous solution. If dialysis is very prolonged, the preparation begins to lose its activity with further decrease in total nitrogen. The activity, however, can be completely restored, as described by Thoai *et al.* (1947), by incubating the partially, but not completely, inhibited enzyme preparation with α -amino acids in a slightly alkaline medium (pH \approx 9). The best preparation contained 0.14 mg nitrogen/ml, and had an activity of 15,700 Roche phosphate units or 1600 King phenol units/mg total nitrogen.

The enzyme was obtained in the dry form without loss of activity by adding pure redistilled acetone to this solution in the cold to a concentration of 80–90 %. It separated as a light coagulated precipitate, which settled overnight in the cold, and was separated by decantation and centrifuging. Various attempts to crystallize this preparation, e.g. by allowing to stand in the cold in concentrated solution with and without spontaneous evaporation in a vacuum desiccator, by careful addition of ethanol and acetone, by allowing to stand at 0° in different concentrations of acetone, by additions of various cations or by addition of ammonium sulphate and other salting-out materials like sodium phosphate and magnesium sulphate, were all unsuccessful.

The total nitrogen content of the dry powders obtained from different preparations was 10–11 % nitrogen. No carbohydrate residue was detected in any of our final preparations. After prolonged

hydrolysis with acid, no reduction of Benedict or Fehling solutions could be detected (cf. Schmidt & Thannhauser, 1943).

Ox-kidney phosphatase. Table 2 shows the activity of ox-kidney preparations during different stages of purification. Our experience with ox kidneys both in England and in France was entirely unsatisfactory. Preparations much weaker in activity than those from dog intestines have always been obtained. The autolysates, as well as the other preparations, are characterized by comparatively low activity and high total nitrogen (generally about 13–15 mg nitrogen/ml).

Table 2 *Activity of ox kidney preparations during stages of purification*

Preparation	Activity	
	Phosphate (Roche units/mg total N)	Phenol (King units/mg total N)
1 Autolysis at room temperature for 3 days in 25 % acetone in presence of 0.1 vol toluene ethylacetate. Autolysate filtered clear, straw yellow	100–135	10–14
2 Crude Albers precipitated by 55 % acetone from above autolysate	32–600	35–65
3 Fractional precipitation by acetone 38–50 %, 0–4°	840–1200	90–130
4 Tryptic digestion and dialysis	2000–3500	250–400

The 38–50 % acetone fraction obtained from crude Albers's preparations contains about 14 % total nitrogen, and is relatively poor in activity. It has a considerable yield of crystals (much more than the corresponding intestinal preparation with higher activity), presumably because of the high phosphate content of this organ. Moreover, the relative instability of the kidney phosphatase during dialysis makes efficient purification of the enzyme by this procedure rather difficult. In this respect it differs from the intestinal enzyme which is more stable, also it is not reactivated to the same extent as the latter by amino acids after partial inactivation through dialysis. This will be discussed in detail elsewhere. Tryptic digestion effected considerable purification of this enzyme.

In Table 3 are given some analytical data of two batches of crystals obtained from two kidney preparations. These were obtained from the first crystallization after thorough washing with cold water and rapid centrifuging. These crystals are soluble with difficulty in water, but are more soluble in dilute alkali or acid. They contained 12–15 % phosphorus, all of which was present as orthophosphate, 4–7 % carbon and very little nitrogen. The fact that the carbon and nitrogen contents are so small and so variable suggests that these are due to organic

impurities and are not chemical constituents of the crystals. The ash consisted entirely of magnesium pyrophosphate. Magnesium was determined microgravimetrically as oxinate by precipitation with 8-hydroxyquinoline (Berg, 1935), and phosphorus determined by the King (1932) method, after hydrolyzing the pyrophosphate into orthophosphate. The

figures shown in the table are very close to those of $Mg_3P_2O_7$. Microtests for calcium and zinc were negative. No ammonia could be detected in the crystals. Comparison with two crystalline forms of magnesium orthophosphate, as shown in Table 3, shows the close similarity of these crystals with either one or the other, with only slight differences due to the presence of traces of organic impurities which are very difficult to remove, and also of water of crystallization which can easily vary when crystals are kept at room temperature. It is concluded that these crystals are magnesium orthophosphate. It appears, therefore, that the alkaline phosphatase has not been crystallized, but is easily adsorbed by magnesium orthophosphate, and, possibly, by other sparingly soluble mineral salts.

Table 3 *Analytical data for crystals obtained from kidney phosphatase*

	Composition			
	Magnesium phosphate		Crystals from phosphatase	
	Theory for $MgHPO_4 \cdot 3H_2O$	Theory for $MgHPO_4 \cdot 7H_2O$	Batch 1 Batch 2	
C*	—	—	7.05	4.3
H*	4.02	3.02	2.36	2.04
N*	—	—	2.24	Trace
P	17.7	12.6	12.5	14.7
Mg	13.9	9.86	10.0	11.27
Ash (as $Mg_3P_2O_7$)	62.1	45.2	45.8	60.2
P/Mg	1.27	1.27	1.25	1.30
	Theory for $Mg_3P_2O_7$		Ash of crystals	
P	28.0		26.5	27.8
Mg	21.6		21.7	22.5

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SUMMARY

1 The purification of alkaline phosphatase from dog intestinal mucosa and ox kidney has been studied by different procedures, and highly active preparations were obtained from mucosa.

2 The crystals which were claimed to be pure alkaline phosphatase have been identified as magnesium orthophosphate, with adsorbed phosphatase.

3 Attempts to crystallize the highly active intestinal phosphatase were unsuccessful.

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Purification of Faecal Alkaline Phosphatase

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The presence of considerable amounts of alkaline phosphatase in dog faeces was first reported by Armstrong, King & Harris (1934). Since then, several attempts have been made to separate and purify this enzyme. Armstrong (1935) described a method by which a very potent phosphatase-containing powder could be obtained from dog faeces within 48 hr. King & Delory (1939) repeated this preparation, but did not succeed in raising its potency

above that achieved by Armstrong. Chen, Freeman & Ivy (1940) concentrated the faecal phosphatase by adsorption on kaolin in an acid medium and elution by alkaline buffer. Their enzyme preparations, however, were less active than those described by Armstrong.

The present work was undertaken for the purpose of obtaining the faecal phosphatase in the purest condition possible, and to attempt its crystallization.

By the use of several methods of purification, it has been possible to obtain preparations with higher activities than those obtained by Armstrong (1935). Several attempts at crystallization were unsuccessful. With the procedure outlined by Armstrong, we encountered many difficulties, and the preparations obtained were, in the majority of cases, much less active than his. Methods of purification have been studied, and an improved procedure is described.

EXPERIMENTAL

The main features of the method adopted were those originally described by Armstrong (1935). The following precautions, modifications and additions, some of them taken from the procedures used for kidney and intestinal phosphatase by Abul-Fadl, King, Roche & Thoai (1949) have been found useful: (1) Avoidance of any change of the pH towards the acid side, as the faecal phosphatase is very sensitive to acid, even at 0°. (2) Fractionation of the aqueous extract with $(\text{NH}_4)_2\text{SO}_4$. (3) Decolorization with minimum loss of activity. (4) Removal of inactive protein material from the final enzyme concentrates by short tryptic digestion followed by careful electro dialysis. The whole procedure is outlined below.

Estimation of activity was in terms of King phenol units as described by Abul Fadl *et al.* (1949).

A Preparation of a clear extract from the faeces

About 1 kg. of dog faeces, collected within 24 hr. of being passed, is placed in a beaker together with cracked ice, and tap water added to make the total volume about 2 l. The contents are stirred thoroughly till uniform and then strained through a wire sieve. The sludge is tested by litmus paper and made alkaline with ammonia. It is then poured into a fluted filter paper and left in the ice box to filter overnight. The filtrate, if not absolutely clear, should be treated with 5% kieselguhr or kaolin and refiltered with suction.

The dark brown filtrate, which should be free from any turbidity, shows an activity of 100–150 King phenol units/ml. and contains about 1.2–0.6 mg. protein N/ml. With a few drops of toluene added, the solution retains its activity for several days at 0°. To assure a high degree of activity, faeces should be collected from healthy, well fed dogs, discarding any samples which have become dry.

It will be noted that preliminary autolysis, as in the case of kidney and intestine, is here unnecessary since the faecal phosphatase is readily obtained in a soluble form.

B Precipitation with ammonium sulphate

Preliminary experiments showed that precipitation with acetone at this stage, as was used in the case of kidney and intestinal preparations, gives preparations of poor activity whilst MgSO_4 , even in full saturation, was of little use in salting out the enzyme. Half saturation with $(\text{NH}_4)_2\text{SO}_4$, however, precipitates about 90% of the phosphatase, while with 0.8 saturation precipitation is almost complete. To each 100 ml. clear filtrate, cooled to 0°, 40 g. $(\text{NH}_4)_2\text{SO}_4$ (A.R.) are added with stirring. On standing for 10–15 min. in the cold a precipitate is formed which rises to the surface. Most of the clear underlying liquid can be removed by siphoning,

and the precipitate readily collected and separated by rapid centrifuging.

The use of acetone at this stage to help salting out, as described by Armstrong (1935), leads to much loss of activity in the aqueous acetone solution. The loss in some experiments amounted to about 40%.

The precipitate thus obtained gives a dark brown turbid solution. The activity is about 600–750 units/ml., with a high protein N amounting to as much as 6.5 mg. N/ml. in some experiments.

C Decolorization by activated charcoal

This is the most critical step in the purification of the faecal phosphatase. Much enzyme activity may be lost if the steps are not carefully controlled. The difficulties encountered are due to the following: (1) The faecal enzyme is unstable in slightly acid medium even in the cold, but for effective decolorization the medium must be slightly acid. (2) Adsorption of the enzyme on the activated charcoal cannot be reversed.

Glycerophosphate buffer adjusted with acetic acid to pH 5.2–5.4 and containing 0.5M MgSO_4 is the best medium for effective decolorization with removal of inactive protein material and minimum loss of enzyme activity. β -Glycerophosphate appears to protect the enzyme against inactivation due to changes in pH, while Mg increases the enzyme affinity for substrate, thus minimizing the risk of adsorption on the charcoal.

The precipitate obtained from B is dissolved in 150 ml. 0.02M sodium β -glycerophosphate solution previously adjusted with dilute acetic acid to pH 5.2–5.4, and 15g. crystal line $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in the solution. The procedure is conducted in the cold at about 0°. Activated charcoal (15 g.) is now added and the mixture is well stirred for 10 min. It is then filtered through a folded filter paper previously prepared by passing through it 1% charcoal suspension in water. The filtrate is almost colourless, or only slightly coloured, and shows an activity of 570–700 units/ml.

The solution is transferred into collodion or cellophane bags of 50 ml. capacity and left to dialyze against distilled water overnight to eliminate most of the Mg, in the presence of which inactivation is likely to occur, and has been observed in many cases. The dialyzed solution, increased in volume by osmosis, is now subjected to further purification as it still contains a lot of inactive protein.

D Digestion with trypsin

The effect of tryptic digestion on purification of phosphatase has been discussed by Abul Fadl & King (1949). A potent commercial trypsin (Armour Ltd.) is thoroughly mixed with the enzyme solution in a concentration of 0.2%. The pH is adjusted to 8.0 with Na_2CO_3 and the mixture left to stand in a thermostat at 37° for 3–4 hr. At the end of this time 5% kaolin is added, stirred for 10 min. and then separated by centrifuging. This procedure is twice repeated in order to eliminate any residual trypsin. If any colour is still present, it can be removed at this stage by a second charcoal treatment (1%), which will also help in removing traces of trypsin, with practically no loss in enzyme activity. The medium need not be acidified in this operation since the colour is only slight and complete decolorization can be easily effected.

The activity now ranges between 400 and 500 units/ml and the protein N from 0.2 to 0.3 mg N/ml. Thus a high degree of purification has been achieved at this stage, but the preparation is still rich in non protein N, which can be eliminated by prolonged dialysis in collodion or cellophan bags against running distilled water.

E Further purification by electrodialysis

Kutscher & Wolbergs (1935) claimed to have obtained the prostatic phosphatase in a high state of activity by long electrodialysis. On the other hand, Albers & Albers (1935) reported a continuous loss of activity in kidney phosphatase preparations subjected to electrodialysis.

We have studied the behaviour of kidney, intestine and faecal phosphatase towards electrodialysis and the detailed results will be published later. With the faecal phosphatase preparations we have been able to attain a further degree of purification by virtue of the denaturation of the remaining proteins which occurs during the electrodialysis.

The apparatus described by King & Dolan (1934) has been used. The clear faecal phosphatase solution is placed in a 150 ml capacity cell, and distilled water is run continuously in the two parchment thimbles containing the electrodes. The current is kept low (10–20 ma) throughout the experiment which lasts usually for 24–30 hr. Care must be taken to keep water running in the thimbles and to stir the dialyzed solution continuously. If a current of N_2 is used for stirring, it must be slow, so as not to form excessive froth which causes surface inactivation of the enzyme. Drops of chloroform are added as a preservative and to minimize frothing.

No physical change is observed in the enzyme solution during dialysis. The activity and protein N, which are tested regularly, show no significant variation throughout the experiment. The enzyme solution remains clear, and only starts to show turbidity near the end of the experiment when diminution in activity is also noticed. On the other hand, turbidity is always found inside the cathode chamber, owing to elimination of nitrogenous and other impurities. If the dialysis is unduly prolonged or the water circulation is stopped, the enzyme solution starts to show turbidity, and precipitation may occur with complete loss of activity.

F Precipitation with acetone

At the end of electrodialysis there is no change of activity in relation to the protein N, and the solution is perfectly clear. The enzyme solution is now cooled to 0° and pure cold acetone is added to a concentration of about 60%. A flocculent precipitate collects at the bottom on leaving to stand in the cold. This can be separated by pouring off the supernatant fluid, centrifuging and washing with pure acetone.

The precipitate thus obtained, however, is not now completely soluble in water. It seems that part of the proteins has been denatured. Distilled water (20 ml) is now thoroughly stirred with the acetone precipitate, allowed to stand in the cold for about 30 min, and any insoluble part spun down. The clear supernatant fluid is reprecipitated with 60–80% acetone in the cold, separated and washed with acetone by centrifugation. It is finally dried over $CaCl_2$ or soda lime in a vacuum desiccator.

The precipitate dries in thin, almost transparent scales. It can be pulverized into a white or buff coloured powder showing an activity of 1166–1300 units/mg N. The total N is 10–11% and protein N is 5.6–6.2%. The yield is 0.2–0.4 g/kg faeces.

DISCUSSION

The activity/mg dry powder is of the order of 95–120 units which corresponds to 190–240 King-Armstrong units. This activity is higher than that recorded by Armstrong (1935), whose best preparation was of about 185 King-Armstrong units.

These purified faecal phosphatase preparations are similar to the intestinal preparations described by Abul-Fadl *et al* (1949) in their nitrogen content and in the absence of any detectable carbohydrate residue. On the other hand, there is a tendency to get more active enzyme preparations from the mucosa since our best faecal preparation shows an activity of 1300 units/mg nitrogen as compared with that from mucosa which was about 1600 units/mg nitrogen. In general, it has been noticed that the most active purified preparations were always obtained from starting materials which were originally of a very high order of activity. This suggests that the enzyme may be originally present in the tissues in quite different amounts or degrees of activity.

As in the case of intestinal phosphatase, repeated attempts at crystallization of the purified faecal enzyme have been unsuccessful.

SUMMARY

1 A method for the purification of faecal alkaline phosphatase is described.

2 Amorphous phosphatase preparations of very high activity have been prepared, but all attempts to crystallize them have failed.

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Purification of Alkaline Phosphatase by Tryptic Digestion

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After fractional precipitation of kidney, intestinal and faecal phosphatase with acetone in the cold, further attempts at purification by either repeated acetone or ammonium sulphate fractionation were ineffective. Treatment with aluminium hydroxide was likewise of little use, and there was considerable loss of enzymic activity. Digestion with trypsin, at this stage, proved to be of great advantage in eliminating inactive proteins which are otherwise difficult to remove.

In most of the current procedures for the preparation of phosphatase, autolysis is used as an indispensable stage in the liberation of the enzyme from the tissues. Ehrenward (1933) discovered that the activity of alkaline phosphatase is not affected by trypsin. Kutscher & Pany (1938) found later that acid phosphatase is resistant to papain. Schmidt & Thannhauser (1943) used artificial proteolysis in the form of tryptic digestion for bringing the calf intestinal phosphatase into solution. Fischer & Greep (1948) have recently reported on an 'activation' of the alkaline phosphatase by trypsin. Thus the tryptic digestion of crude phosphatase preparations is well known, but its effect on highly concentrated purified enzyme preparations has not been well investigated. For this reason, it seemed desirable to conduct such a study to find out the best conditions for purification with minimum loss of enzyme activity.

EXPERIMENTAL

Enzyme solutions from ox kidney and dog intestinal mucosa treated by fractionation with acetone in the cold, and dog faecal phosphatase prepared by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ and charcoal decolorization, have been used. A very potent trypsin powder (U S P X, Armour Laboratories, Chicago) has been used throughout. The enzyme solutions were adjusted to pH 8 by Na_2CO_3 solution and kept at 37° with various concentrations of trypsin powder. Activities expressed in King phenol units in relation to protein N by micro Kjeldahl (see Abul Fadl, King, Roche & Thoai, 1949) have been determined in the presence of Mg^{++} on samples withdrawn from the incubated enzyme solutions at regular intervals.

After tryptic digestion there is always a considerable amount of non-protein N, which is best eliminated by prolonged dialysis.

RESULTS AND DISCUSSION

Table 1 shows the effect of tryptic digestion on faecal and intestinal phosphatases. An initial increase of the enzyme activity in relation to protein nitrogen

during the first few hours of incubation is followed by a gradual fall. It is, therefore, inadvisable, with 0.1–0.2% trypsin, to incubate for longer than

Table 1 *Effect of trypsin digestion on partially purified alkaline phosphatase*

Enzyme solution	Tryp- sin con- centration (%)	Time of incuba- tion at 37° (hr)	Protein N (mg/ml)	Phosphatase activity	
				(Units/ml)	(Units/mg protein N)
Intestinal	0.1	0	0.20	300	1500
		2	0.18	300	1720
		4	0.16	255	1569
		6	0.16	255	1569
		8	0.16	240	1480
Intestinal	0.2	0	0.17	315	1852
		1.5	0.14	293	2192
		3	0.13	243	1868
		4.5	0.12	225	1800
		6	0.12	200	1660
Faecal	0.5	0	0.40	240	600
		2	0.28	240	857
		4	0.24	200	833
		6	0.24	185	770
		24	0.24	144	600
		30	0.24	144	600*
		48	0.24	126	525*
		60	0.24	102	425

* More trypsin added

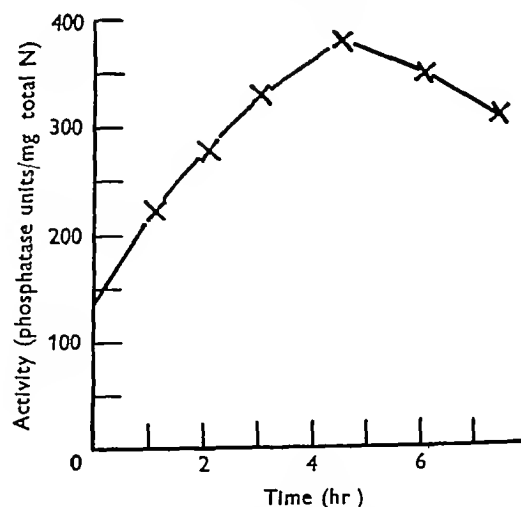


Fig 1 *Effect of tryptic digestion on kidney phosphatase 0.1% trypsin, 37° , pH 8.0*

2–3 hr at 37° . This depends on the degree of contamination of the enzyme with inactive protein. With kidney preparations (Fig 1), however, a longer

incubation is required (4–5 hr) for the maximum activity relative to protein nitrogen to be attained, since these are more contaminated by inactive protein than either faecal or intestinal preparations. It is interesting to note that trypsin does not appear to act on the protein part of the enzyme, since the protein nitrogen of the incubated enzyme solution remains constant after a certain time, and is not affected by any further incubation, even if more trypsin is added. The decline in enzyme activity appears to be due to inactivation of the enzyme, when incubated in alkaline buffer, rather than to direct trypsin action on the enzyme itself. This inactivation was pointed out by Fischer & Greep (1948) to be much more extensive in the absence of trypsin than when it is present. Thus trypsin, in addition to

digesting contaminating proteins, seems to protect the enzyme to a certain extent against inactivation during incubation in alkaline buffer. Excess of trypsin, however, does not seem to increase the degree of protection of the enzyme, on the contrary, it seems to decrease it, or to cause inhibition of the phosphatase.

SUMMARY

1 Further purification of the alkaline phosphatase, beyond that attainable by fractional precipitation and salting-out procedures, was obtained by incubation with 0.1–0.2% trypsin at 37° for a period of not more than 4 hr.

2 Addition of more trypsin, or incubation for longer periods, resulted in loss of enzyme activity.

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Reactivation of Alkaline Phosphatases after Dialysis

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Albers, Bayer, Bohnenkamp & Muller (1938) ascribed the inactivation of the kidney alkaline phosphatase through prolonged dialysis to a separation of the phosphatase into an inactive protein apoenzyme and a dialyzable coenzyme or prosthetic group. These workers showed that this coenzyme cannot be magnesium. It appeared to be a labile organic substance, which could be obtained by incubating the holoferment at 100° for a few minutes in a neutral medium. This treatment destroyed the apoenzyme while not affecting the coenzyme. By mixing the latter with an enzyme preparation partially inactivated by dialysis or previous incubation in an acid medium, active phosphatase solutions were obtained. On combining the dialyzable liquid with the inactivated protein solution of dialyzed kidney preparations, with the addition of magnesium, Albers (1939) claimed to have obtained an almost 100% reactivation. von Euler & Hahn (1947) described a partial reactivation of intestinal phosphatase, inactivated by dialysis at pH 4.5, by the addition of boiled enzyme solution. Cloetens (1941*a, b*, 1942), after repeating Albers's experiments, came to the conclusion that the activating action of a heated enzyme solution (Kochsaft), or the dialysate

liquid, was not due to the presence of a cophosphatase, but more probably to a zinc or cobalt ion, which is present in very small amount and which forms a dissociable complex. After studying the influence of different metallic ions, as well as substances which form weakly dissociable combinations with metals, Cloetens concluded that alkaline phosphatase is composed of a slightly active or completely inactive part, EG_2^1 (i.e. protein + metal-combining groups), and the two metals magnesium and zinc. This postulate was supported by the work of Hove, Elvehjem & Hart (1940), who used zinc salts in combination with amino-acids and with protein split products (from autolyzed mucosal tissue) to activate dialyzed intestinal phosphatase. Thoai, Roche & Roger (1947) reported the inactivation of intestinal phosphatase by prolonged dialysis at 37°, and its complete reactivation by incubation with amino-acids in alkaline medium and determination of the enzyme activity in presence of certain inorganic ions. Comparing these findings with that of Albers, it would appear possible that the dialyzable prosthetic group may be a combination of a metal other than magnesium (possibly zinc) with a compound containing amino-acid groups.

In the present investigation an attempt has been made, using ordinary dialysis as well as electro-dialysis, to obtain a clearer conception of the nature of the dialyzable factors indispensable for the activity of alkaline phosphatase. It has been found that magnesium and a nitrogenous non-protein dialyzable substance, which migrates towards the cathode during electro-dialysis, are responsible for activating the apoenzyme. No evidence for the identity of this dialyzable substance with simple α amino acids, or for the presence of zinc, has been obtained. Differences among alkaline phosphatase preparations from different sources have been found, and the results are discussed.

EXPERIMENTAL

King & Dolan's (1934) electro-dialyzing apparatus has been used throughout. It consists of a glass cell of suitable capacity containing two units of electrode chambers immersed in the enzyme solution. Each unit consists of a parchment dialyzing thimble containing the electrode surrounded by distilled water. Because of the shortage of platinum this has been used only for the cathode, while a carbon electrode has been found quite satisfactory as an anode. The two electrode chambers were either connected together, as when water is circulated through them, or left unconnected, according to the requirements of the experiment. A mechanical stirrer was used, since the bubbling of N_2 was found to cause some inactivation due to frothing. A mercury vapour lamp transformer was used as the source of direct current. In some cases a 6 V battery was used. An ammeter was included in the circuit. The current passing through the enzyme solution was as low as 1.5 ma and was never allowed to exceed 10 ma.

Enzyme solution and determination of activity. The solutions being dialyzed always contained the enzyme in its highest available state of purity, prepared and the activity determined according to the methods already described (Abul Fadl, King, Roche & Thoai, 1949, Abul Fadl & King, 1949).

Nitrogen determination. The micro Kjeldahl method was used, and was carried out on 0.2 ml. of the clear enzyme solution on which the activity had been previously determined. For protein N the proteins were precipitated with molybdic acid. Digestion was carried out with 2 ml 50% (v/v) H_2SO_4 containing 1% SeO_2 , a small electric heater being used.

Micro-detection of certain metals (cf. British Drug Houses Ltd. *Book of Organic Reagents for Analytical Use including Spot Tests*). Magnesium was tested for by the *p*-nitro benzeneazo α naphthol test which is sensitive to 2 μg Mg, calcium by the picrolonic acid test which is sensitive to 0.01 μg Ca, and zinc by the resorcinol test, sensitive to 2 μg Zn.

Procedure for dialysis. The enzyme solution was subjected to prolonged electro-dialysis against running distilled water, continuously stirring and using an electric current of 1-3 ma. The enzyme activity was determined at regular intervals, with and without the addition of Mg^{++} to the buffer substrate mixture. As the activity started to decrease, the two electrode chambers were disconnected from each other, and distilled water was very slowly passed through each chamber separately, and collected in separate containers, or if necessary the water circulation was stopped. The current was gradually increased.

The effect of the anode and cathode liquids, as well as certain other substances, was then tried on the enzyme solution which had been partially inactivated by electro-dialysis.

RESULTS

Kidney phosphatase. Table 1 shows the changes in enzyme activity in absence and in presence of magnesium, and in total nitrogen and protein nitrogen, taking place during electro-dialysis of kidney alkaline

Table 1 *Effect of electro-dialysis on the activity of kidney alkaline phosphatase*

(Slow current of running distilled water in the electrode chambers, electric current started with 1 ma and gradually increased to 10 ma.)

Duration of electro-dialysis (hr)	Enzyme activity (units/ml)		mg N/ml	
	Without Mg	With 0.01 N-Mg	Total	Protein
0	56	84	4.6	1.30
2	56	84	4.5	1.30
4	56	84	4.3	1.30
6	56	84	4.2	1.30
8	53	84	4.1	1.30
10	50	84	4.0	1.30
12	46	84	3.9	1.30
14	40	82	3.8	1.28
16	34	80	3.7	1.27
18	28	53	3.6	1.25
20	20	36	—	—
26	6	9	3.4	1.23
30	3	4	—	—
34	2	2	3.2	1.20
36	0.8	0.8	—	—
40	0.3	0.3	3.0	1.20

phosphatase solution. A progressive loss of activity occurs, and it appears that at least two important factors are lost during the course of the dialysis. The first of these is magnesium, the addition of which restores the original activity of the enzyme solution during the first 16 hr of electro-dialysis, although the time depends on the current strength. After that a sudden drop in activity takes place followed by a comparatively rapid inactivation of the enzyme. This suggests the splitting off of another factor which cannot be identical with magnesium since the latter, even in increased amounts, fails to restore the initial enzyme activity. It is noticed that the more inactivation takes place the more this factor is lost, and the less the response of the enzyme towards magnesium until finally the latter has almost no effect on the enzyme activity.

The total nitrogen falls gradually with electro-dialysis. The protein nitrogen remains constant for a time, and then decreases gradually, the solution becoming turbid especially around the cathode chamber. There is a tendency for the protein nitrogen to remain constant again near the end of the dialysis.

Table 2 shows the strong activating power of the clear cathode liquid after filtration from the inactive precipitate. The cathode liquid alone has no activity, but it has considerable activating power when mixed with the apoenzyme, and this is much more pronounced in the presence of magnesium. The three experiments were carried out on the same solution.

Table 2 *Effect of the liquids in the electrode chambers on kidney alkaline phosphatase partially inactivated by electro dialysis*

(These experiments were carried out on a single enzyme solution which was dialyzed in three stages. At the end of each stage the enzyme solution was mixed with the liquids from the electrode chambers collected during the period of dialysis. The water was circulated through the dialyzing thimbles as slowly as possible.)

	Activity (units/ml)	
	Without Mg	With 0.01 M Mg
Stage 1 of dialysis		
Dialyzed enzyme solution (30 hr)	28	53
(+ water, 1 l)		
After 2 hr further dialysis (+ water, 1 l)	20	36
Enzyme solution + anode liquid (1 l)	20	36
Enzyme solution + cathode liquid (1 l)	38	62
Enzyme solution + anode + cathode liquids (1.05 l)	38	61
Cathode liquid alone	0	0
Anode liquid alone	0	0
Cathode + anode liquids	0	0
Stage 2 of dialysis		
Dialyzed enzyme solution (total 35 hr) (+ water, 1 l)	8	11
After 1 hr further dialysis (+ water, 1 l)	7	9
Enzyme solution + anode liquid (1 l)	7	9
Enzyme solution + cathode liquid (1 l)	9	15
Stage 3 of dialysis		
Enzyme solution after prolonged dialysis (+ water, 1 l)	5	5.5
Further dialysis (+ water, 1 l)	0	0
Enzyme solution + anode liquid (1 l)	0	0
Enzyme solution + cathode liquid (1 l)	1.2	5.3

which had been electro dialyzed in three stages. The activation is very marked during the initial stage of electro dialysis, but after prolonged electro dialysis the cathode liquid does not fully restore the initial activity of the enzyme. It seems probable that the apoenzyme is affected by the prolonged dialysis.

The anode liquid, inactive in itself, has neither activating nor inhibiting action when mixed with the enzyme solution being dialyzed.

Table 3 shows the effect of magnesium, zinc, amino acids and cathode liquid added to two enzyme solutions of different initial activities which had been partially inactivated by electro dialysis, singly and

in different combinations. Zinc in 0.0002–0.0005 M concentrations and in presence of 0.01 M magnesium shows a slight activating property, but inhibits in higher concentrations. Its activating effect, however, is very small compared with that of the cathode liquid. A combination of cathode liquid and

Table 3 *Effect of magnesium and zinc salts and amino-acids compared with that of cathode liquid on the kidney alkaline phosphatase partially inactivated by prolonged electro dialysis*

	A (units/ml)		B (units/ml)	
	No Mg	0.01 M Mg	No Mg	0.01 M Mg
	40	60	13	20
Original solutions	40	60	13	20
After prolonged dialysis	3.0	3.4	2.7	3.0
Effect of zinc salt				
0.01 M	0.0	0.5	0.0	0.7
0.001 M	1.6	5.6	0.7	9.6
0.0005 M	2.0	10.5	1.2	11.5
0.0002 M	2.5	10.5	1.5	11.5
0.0001 M	2.7	5.8	2.0	3.5
Incubation with amino acids				
Glycine (0.001 M)	0	0	0	0
Glycine (0.0001 M)	1.5	1.0	0.7	0.5
Alanine (0.001 M)	0	0	0	0
Alanine (0.0001 M)	2.0	1.5	1.5	1.2
Enzyme solution mixed with cathode liquid (1 l)	28	55	7	18.5
Enzyme solution mixed with cathode liquid, and 0.0002 M-zinc added	6	22.5	2.5	9.4

0.0002 M-zinc has less activating power than the cathode liquid alone. The amino-acids (glycine and alanine) in different concentrations, even after previous incubation with the inactive enzyme, do not exhibit any activating property, on the contrary, they inhibit, especially in presence of magnesium.

Table 4 shows that the activating substance present in the cathode liquid is organic in nature as the ash is not active. It is stable to boiling for 10 min but is destroyed by a slight excess of ammonia.

A cathode liquid with high coenzyme activity contained 3.9 mg total solids/ml and yielded 0.60 mg of ash, mainly alkaline earths. 'Spot' tests for calcium and for zinc in the ash were negative, while a slight positive reaction for magnesium was obtained. The total nitrogen, 0.66 mg/ml, was equivalent to 20% of the organic matter. This is consistent with the possibility of an amino-acid or peptide structure being present. Protein was apparently absent since there was no detectable precipitate with molybdic acid.

Table 4 *Effect of boiling, ashing and other treatments on the activating property of the cathode liquid on the kidney phosphatase*

	Activity (units/ml)	
	No Mg	0.01 M Mg
Kidney phosphatase after 8 hr electro dialysis	12.6	19
Enzyme solution + cathode liquid	20	60
Enzyme solution + boiled cathode liquid	20	60
Enzyme solution + redissolved ash*	12.0	18.5
Enzyme solution + cathode liquid treated with 0.05N ammonia solution	12.6	19.5

* Cathode liquid evaporated to dryness, residue ignited, and ash dissolved in HCl, evaporated on water bath to dryness, then dissolved in water, neutralized and diluted to original volume

Intestinal, faecal and liver phosphatase Table 5 illustrates the effect of electro dialysis on these alkaline phosphatases. The enzyme activity/ml remains almost constant for the first 20 hr, then a

Table 5 *Effect of electro dialysis on intestinal, faecal and liver phosphatases, and the effect of the electrode chamber liquids on the enzyme activity*

	Activity (units/ml)	
	No Mg	0.01 M Mg
Intestinal phosphatase (+ water, 1 l)	140	200
Electro dialysis against running distilled water, 1.5 increased to 10 ma		
After 2 hr	140	200
" 6 "	140	200
" 10 "	130	200
" 16 "	125	200
" 20 "	115	180
" 24 "	90	130
" 28 "	40	55
" 30 "	28	38
Enzyme solution + anode liquid (1 l)	18	27
Enzyme solution + cathode liquid (1 l)	36	45
Dialysis for further 4 hr		
Enzyme solution (+ water 1 l)	0.4	0.4
" (+ anode 1 l)	0	0
" (+ cathode 1 l)	1.2	1.4
" (+ anode + cathode)	0	0
Faecal phosphatase solution (+ water 1 l)	180	240
After 30 hr electro dialysis against running distilled water, 1.5-10 ma	65	95
Enzyme solution + anode liquid (1 l)	34	70
Enzyme solution + cathode liquid (1 l)	75	120
Liver phosphatase solution (+ water 1 l)	96	120
After 20 hr electro dialysis against running distilled water, 5 ma	35	50
Enzyme solution + anode liquid (1 l)	15	22
Enzyme solution + cathode liquid (1 l)	45	65

sudden drop takes place. The cathode liquid showed a slight activating effect, but not as much as that noticed in the case of the kidney phosphatase. In some cases the cathode liquid was without any detectable activating effect, and the dialyzed enzyme lost its activity without any evidence of separation of a coferment, contrary to what was always found with the kidney phosphatase.

On the other hand, the anode liquids showed a tendency to inhibit the intestinal, faecal and liver phosphatases. The pH of these liquids ranged from 2.3 to 4.7, and the inhibitory effect was more pronounced with the more acid solutions. The kidney enzyme was not sensitive to the same solution.

Effects of magnesium on phosphatases Fig 1 shows the effects of different magnesium concentrations on the activity of crude and purified intestinal and kidney phosphatase preparations. The enhancing

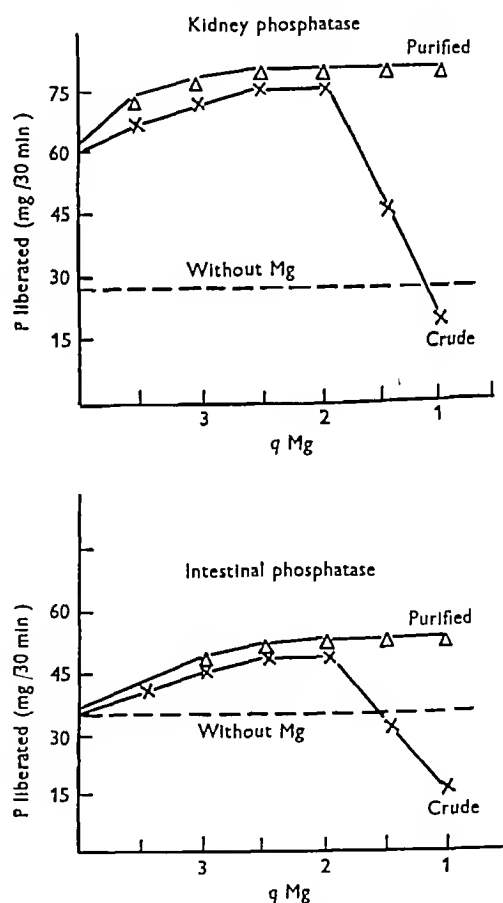


Fig 1 Effect of Mg on crude and purified alkaline phosphatases. Hydrolysis of 0.005M sodium phenyl phosphate in carbonate bicarbonate buffer, pH 10, $q\text{Mg} = -\log[\text{Mg}^{++}]$

action of magnesium on kidney phosphatase is much more pronounced than that on the intestinal enzyme. With crude enzyme preparations there is an optimum concentration of magnesium beyond which the enzyme is inhibited. With purified enzyme

preparations, on the other hand, excess of magnesium has no such inhibitory effect

Effect of amino acids Table 6 shows the effect of amino-acids on kidney and faecal enzymes. Both non-dialyzed kidney and faecal enzymes are activated by some amino-acids. The dialyzed kidney enzyme shows only feeble activation with cystine and cysteine, while glycine does not activate. The

Table 6 *Effect of amino acids on faecal and kidney alkaline phosphatase before and after dialysis*

(Ordinary dialysis in cellophan bags at room temperature against distilled water)

	Activity (units/ml)	
	No Mg	0.01 M-Mg
Non dialyzed kidney phosphatase*	17.3	26.3
Glycine (0.002M)	27.8	30.5
Cysteine (0.0005M)	27.0	29.3
Histidine (0.001M)	27.0	32.5
Cystine (0.001M)	27.0	31.0
Dialyzed kidney phosphatase* (36 hr)	9.0	10.5
Glycine (0.0002M)	8.5	9.0
Cystine (0.005M)	10.6	12.4
Cysteine (0.0005M)	10.0	11.5
Non dialyzed faecal phosphatase*	82.5	112.0
Glycine (0.0002M)	96.0	125.0
Dialyzed faecal phosphatase* (36 hr)	14.0	15.2
Glycine (0.0002M)	35.7	46.5
Alanine (0.0005M)	36.0	45.8
Cysteine (0.0005M)	14.6	16.5
Cystine (0.0005M)	14.6	16.5
Histidine (0.0005M)	45.7	62.5
Histidine (0.001M)	45.7	62.0
Histidine (0.002M)	45.0	62.0
Tyrosine (0.001M)	20.3	25.6
Tryptophan (0.001M)	17.8	22.0

* Different preparations

dialyzed faecal enzyme, on the other hand, shows a marked activation with amino-acids, that with histidine being especially noteworthy. This does not seem to be lessened with increasing concentrations of histidine up to 0.002M. Cystine and cysteine, on the other hand, have no effect and tryptophan only a slight activating effect.

Effect of formaldehyde Table 7 shows the effect of formaldehyde on the phosphatases obtained from different tissue autolysates. By inclusion of 0.1 ml of 20% neutral formaldehyde solution in the buffer-substrate enzyme mixture (without previous incubation of the enzyme with the formaldehyde solution) a 95–100% inhibition was attained with all tissue alkaline phosphatases, except those of intestinal mucosa and faeces, where the inhibition was 70%.

Attempts were made to reactivate a kidney alkaline phosphatase that had lost most of its activity through formaldehyde treatment. Directly after adding the formaldehyde solution to the enzyme

preparation a slight excess (over the theoretical to combine with the formaldehyde) of ammonia solution was added. The activity was then determined,

Table 7 *Effect of formaldehyde on the alkaline phosphatase of tissue extracts*

	Activity (units/100 ml)		Inhibition (%)
	Without formal- dehyde	Formal- dehyde (0.5%)	
Ox kidney	138.5	5	96
Human kidney	77.3	1.5	97
Adrenal glands	165	0	100
Dog bile diluted 1:200	28.5	0	100
Bone (young rabbit)	162	1.5	99
Dog faecal	75	24	68
Human intestinal	165	60	64
Liver	97	0	100
Pancreas	9	0	100
Spleen	120	0	100
Thyroid	47	0	100

and the effects of different amino-acids (glycine, alanine, histidine, arginine, tyrosine and tryptophan) as well as of the cathode liquid were tried. It was not possible to restore the activity by means of any of these substances.

DISCUSSION

These experiments support the hypothesis of Albers *et al.* (1938) and of Albers (1939) that phosphatase consists of an apo- and a co enzyme. This is well illustrated with kidney phosphatase by a progressive loss of enzyme activity during electro dialysis, due to splitting off a factor (or factors) essential for enzyme activity which migrates towards the cathode. Thus the liquid in the cathode chamber shows strong reactivating properties when added to the holoferment which has been partially inactivated by electro dialysis. The liquid in the anode chamber, on the other hand, has no action on the enzyme activity. The anode and cathode liquids possess no enzymic activity either alone or combined.

The activating effect of the cathode liquid is mainly due to two factors: (A) magnesium (which is possibly replaceable by other metals), which can be easily split off and is responsible for the whole activating property of the cathode liquid during the early stages of electro dialysis, especially with weak currents, (B) an organic factor (possibly nitrogenous), which is not easily detachable from the apo-enzyme, but splits off quickly with strong currents, or suddenly after prolonged ordinary dialysis. This factor withstands prolonged boiling, but is readily destroyed when treated with ammonia, excess alkali or acid. It is not identical with any of the amino-acids, since the latter do not exhibit a similar effect.

Magnesium seems to be essential for the activation of the holoferment. When the latter is carefully freed from magnesium and then added to the partially inactivated enzyme, it will produce no effect, but when magnesium is added as well the phosphomonoesterase activity of the mixture is much more than could be produced by adding magnesium alone. On the other hand, magnesium alone has no effect on the completely inactive enzyme, but in presence of the organic factors the activity is restored, although in no case to the initial level. Complete inactivation of the enzyme is probably accompanied by denaturation of the apoenzyme.

Under the conditions of these experiments this organic factor is essential for the reactivation of the dialyzed holoferment from ox kidney. It does not require any previous incubation, nor has it any inhibitory effect when present in excess. In contrast, it has no such action on the non dialyzed kidney enzyme, nor on the electro-dialyzed inactive enzyme prepared from liver or intestine. A possible explanation is that in the case of the liver and intestinal enzymes this factor is so strongly bound to the apoenzyme that the latter is inactivated by prolonged dialysis before any splitting of the coenzyme takes place.

These facts seem to indicate that the kidney phosphatase consists of three main components which are indispensable factors for the enzymic activity: a specific protein portion, a specific dialyzable organic group or groups, and magnesium. Magnesium produces its effect only in the presence of the dialyzable organic group or groups.

The two-metal theory involving zinc seems to be highly improbable since no evidence that zinc is indispensable has been obtained.

The behaviour of faecal, intestinal and liver phosphatases during dialysis has been found to differ from that of the kidney enzyme under the same experimental conditions.

The following are the results of a great number of experiments which have been carried out in this connexion.

(1) By ordinary dialysis in cellophan bags against distilled water, or feebly alkaline water at room temperature, it was observed that the kidney enzyme progressively decreased in activity. After 24 hr dialysis the activity was greatly decreased, but it could be restored to the original level through the addition of optimum concentrations of magnesium. On further prolonged dialysis the activity still declined and after 48 hr a marked loss of phosphatase activity took place. The enzyme was still activated by magnesium, but this could no longer restore the activity to its original value. The faecal, intestinal and liver enzymes, on the other hand, were markedly stable under the same conditions. Although a limited decrease in activity took place

magnesium could still restore the original activity. Beyond this no further decline in activity took place, and the enzymes remained stable during 72 hr dialysis in one experiment. With more prolonged dialysis activity was maintained for about 8–10 days at room temperature, following which it rapidly declined. This phenomenon can be explained on the basis of the previously mentioned postulate that the coenzyme, while very strongly bound to the apoenzyme in the case of faecal, intestinal and liver phosphatases, can be comparatively easily split in the case of the kidney enzyme.

(2) On electro-dialysis the kidney enzyme was comparatively more stable than the other phosphatases. This could be partially accounted for, in many cases, by the marked sensitivity of these enzymes to anions. The anode liquid inhibited these enzymes, while it was quite harmless to the kidney enzyme. If the water circulating in the anode chamber was stopped during electro-dialysis, the intestinal phosphatase was inhibited very quickly (even if dialysis was carried out at 0°), while the kidney enzyme, on the other hand, was not affected.

Although several factors have been known to influence the degree of activation of phosphatase by magnesium (e.g. concentration of magnesium, nature of substrate and its concentration, degree of enzyme purity, concentration and age, cf. Fischer & Greep, 1948), yet there have been strong indications that certain phosphatases are more activated by magnesium than others. Armstrong (1935) and Schmidt & Thannhauser (1943) described a 25% increase in the activity of faecal and intestinal phosphatases with optimum magnesium concentration. Drill, Annegers & Ivy (1944) also reported slight enhancement by magnesium of the elevated serum phosphatase during hepatic damage. Our findings concerning faecal, intestinal and liver phosphatases are in agreement with those of these authors. The kidney enzyme, on the other hand, has always been found to be much more activated by magnesium than either faecal, intestinal or liver phosphatases. This can again be explained on the basis of variation of affinity between the apoenzyme and its coenzyme and magnesium, in different phosphatases. When this affinity is very strong, the enzyme, as it naturally occurs, will be almost saturated with its requirements of magnesium, and hence the effect of any added amount will be only slight. On the other hand, when this affinity is weak, the enzyme will lose its magnesium readily during its extraction from the tissues and by mere ageing or by dilution, and thus the effect of added magnesium will be pronounced (cf. Fischer & Greep, 1948).

Amino acids are shown here to activate both kidney and intestinal non-dialyzed enzymes. After prolonged dialysis of these enzymes, however, the effect of amino-acids on kidney enzyme was almost

negligible, while their strong activating influence was still persistent with the intestinal enzyme. This suggests that amino-acids exert their effect through certain groups in the coenzyme, which are easily split from the kidney enzyme while being relatively stable in the intestinal enzyme during dialysis.

The activation of kidney phosphatase by cysteine has been similarly reported by Williams & Watson (1940), who found that cysteine in low concentrations activates the alkaline phosphatase of bone, while with higher concentrations inhibition occurs.

On the other hand, Waldschmidt Leitz, Schaffner & Bauer (1933), Bodansky (1936*a, b*) and Thannhauser, Reichel & Grattan (1937) reported that thiol compounds such as cysteine decrease the phosphatase activity.

With the dialyzed faecal phosphatase preparation in Table 6 both cysteine and cystine failed to reactivate the enzyme, while other amino-acids did so, especially histidine whose effect was marked up to a concentration of 0.002M. Bodansky (1948) has recently reported the inhibitory effect of L-histidine on the activity of intestinal, bone and kidney phosphatases using higher concentrations (0.0064M) than used here. He also pointed out that the basic amino-acids, L-histidine and L-lysine exert a greater inhibitory effect on rat-bone and kidney phosphatases than on intestinal phosphatase, whereas the reverse holds for the dicarboxylic L-glutamic acid.

The effect of formaldehyde on the different alkaline phosphatases provides a further distinction between kidney phosphatase and the intestinal and faecal enzymes. This phenomenon was previously noticed by Gould (1944). Bodansky (1937) also showed a difference between the intestinal phosphatase and other tissue phosphatases in connexion with their behaviour towards bile salts. The intestinal enzyme is less sensitive towards this inhibitor than the other phosphatases. It seems that the amino groups of the

apoenzyme are essential for the phosphatase activity. Attempts to reactivate the formaldehyde-inhibited enzyme by addition of ammonium salts, amino-acids or cathode liquid were unsuccessful.

Owing to the fact that the kidney alkaline phosphatase has not yet been obtained in a satisfactory degree of purity, an investigation of the nature of the dialyzable coenzyme is not yet possible.

SUMMARY

1 The mechanism of enzyme inhibition during electrodialysis of faecal, intestinal, kidney and liver alkaline phosphatases has been studied.

2 The migration of a substance with strong reactivating properties towards the cathode during dialysis of the different phosphatases was most clearly demonstrable with the kidney enzyme. The properties of this substance have been investigated and compared with certain other substances.

3 No evidence in favour of the two-metal theory involving zinc could be obtained.

4 The presence of three main components seems to be indispensable for the normal alkaline phosphomonoesterase activity: a specific protein apoenzyme, an organic dialyzable coenzyme, and magnesium.

5 The activation of the enzyme by magnesium, and possibly certain other metals and amino-acids, takes place only in the presence of the dialyzable coenzyme.

6 The differences among the alkaline phosphatases of different origins are probably due to different affinities between the apoenzyme and its coenzyme, and possibly to slight variations in the nature of the apoenzyme in different phosphatases.

7 The apoenzyme appears to contain amino groups, which are essential for the phosphatase activity.

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The Action of Tyrosinase on Monophenols

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The many studies of tyrosinase have left unsolved the problem of the relationship between the so-called 'monophenolase' and 'catecholase' or 'polyphenolase' functions of this enzyme. It has seemed improbable that the same enzyme should effect two such dissimilar reactions as the introduction of a second hydroxyl group into the phenol ring and the dehydrogenation of an *o*-diphenol to give an *o*-quinone, yet the postulation of an independent specific monophenolase must remain under grave suspicion until fractions are obtained in which the relative monophenolase/catecholase activity is greater than in the crude mushroom juice. The alternative postulate, that the oxidation of monophenol is a secondary non-enzymic reaction, due to hydrogen peroxide or *o*-quinone formed from traces of *o*-diphenol which are presumed to be present, was first made by Onslow & Robinson (1928). It was criticized by Pugh (1929) and by Bordner & Nelson (1939), and a reconsideration of the evidence has led Nelson & Dawson (1944) to dismiss this possibility. The latter authors conclude that the monophenolase and catecholase functions are originally exercised by the same enzyme or enzyme complex, but that a centre in the enzyme which is essential for the monophenolase function, but not for the catecholase function, is (a) rather unstable towards the fractionation techniques which have been used, so that monophenolase activity is easily lost, and (b) inactive towards monophenol unless *o*-diphenol is being simultaneously oxidized by the enzyme, so that there is an induction period only relieved either by the slow build up in *o*-diphenol concentration initiated by spontaneous oxidation, or by the addition of *o*-diphenol to the system.

The first product of oxidation of monophenol is believed to be the corresponding *o*-diphenol, although the *o*-diphenol has only been isolated in the case of tyrosine \rightarrow dihydroxyphenylalanine (Raper, 1926). The *o*-diphenol so formed is then also available as substrate to the enzyme, and may be oxidized to the quinone and beyond in the case of the primary substrates most studied (tyrosine, phenol, *p*-cresol) to a stage involving a further uptake of oxygen of 1 atom/mol or more. The composition of the reaction mixture thus becomes quite complex with several different reactions proceeding simultaneously, but, in the studies of 'monophenolase' activity so far made, the monophenolase activity has been assessed

in terms of the total oxygen uptake in this complex system. Keilm & Mann (1938) first reported the catalyzed oxidation of ascorbic acid in solutions containing tyrosinase and catechol, and Miller & Dawson (1941) have made use of this in their 'chronometric' method for measuring catecholase activity. The presence of ascorbic acid does not affect the catecholase activity of the enzyme, but *o*-quinone formed is then almost instantaneously reconverted to *o*-diphenol by the ascorbic acid, and is not detectable in the reaction mixture until all the ascorbic acid has been oxidized. This suggested that a study of the oxidation of monophenols by the enzyme in the presence of ascorbic acid might be of value. Under such conditions *o*-diphenol might be expected to accumulate, and the reaction mixture to remain uncomplicated by the accumulation of pigmented oxidation products of unknown constitution. The conditions would in fact be suitable for a study of the primary reaction, monophenol \rightarrow *o*-diphenol. The present paper describes methods for the estimation of *o*-diphenol in reaction mixtures containing initially monophenol, tyrosinase and ascorbic acid, and results obtained with these methods in studies of the conversion of monophenol to *o*-diphenol in the cases of tyrosine, phenol, *p*-cresol and 4,5-dimethylphenol*. The experiments were carried out in the hope that the data obtained might throw more light on the vexed question of the mechanism of monophenol oxidation by tyrosinase.

METHODS

Enzyme preparation The enzyme was prepared from the common cultivated mushroom, *Psalliota campestris*, by the method of Keilm & Mann (1938). The second fractional adsorption on $\text{Ca}_3(\text{PO}_4)_2$ in this procedure gave two fractions, the first of which, having $Q_{0.1}$ (catechol)† and $Q_{0.2}$ (*p*-cresol) values of c. 70,000 and 35,000, respectively, when freshly prepared, was used in all the experiments described in this paper. It was stored in the refrigerator under toluene, and had been so kept for 2 years when the work was begun. During this time its activity towards *p*-cresol had decreased by about one third, whilst the activity towards catechol appeared to have remained unchanged.

* This compound is strictly named '3,4-dimethylphenol'. The alternative name '4,5-dimethylphenol' is used throughout this paper to emphasize the relationship of the phenol to the *o*-diphenol, 4,5-dimethylcatechol, formed from it.

† All $Q_{0.2}$ values are in $\mu\text{l O}_2/\text{mg dry wt/hr}$.

Conditions in tyrosinase experiments The reaction mixtures in all cases contained 0.1M-phosphate buffer (pH 7.0), and the temperature was 25°. The reactions were in general carried out in open vessels vigorously aerated by a stream of air for the provision of samples for *o*-diphenol and ascorbic acid estimation, and in the Warburg manometric apparatus with identical reaction mixtures, using a 2.00 ml. fluid phase and the standard technique, for the measurement of O_2 absorption. In the early experiments, with tyrosine as substrate, excellent agreement was obtained between the O_2 requirement calculated to correspond with the changes in ascorbic acid and *o*-diphenol concentration, and the O_2 uptake measured in parallel experiments. Later it was found in certain cases, viz. with phenol as substrate at the highest concentration used (0.1M) and also with 4.5 dimethylphenol at quite low concentrations, that the oxidation of ascorbic acid and formation of *o*-diphenol was equivalent to a much smaller O_2 uptake than was observed in the parallel experiment. The difference could in neither case be attributed to a deficiency in aeration in the open vessel experiment, but, when this difference was observed, a rather persistent froth was always present above the surface of the reaction mixture in the open vessel, and the most likely explanation is that a major fraction of the enzyme was adsorbed in this froth and rendered ineffective by removal from the solution. To overcome this difficulty when it arose, manometric experiments only were carried out, in duplicate. Individual manometers were removed from the bath at different times after the beginning of the reaction, and their reaction mixtures immediately analyzed for ascorbic acid and *o*-diphenol. Composite curves were constructed from the data to show the changes in the reaction mixture with time.

Estimation of o-diphenols

It was desired to estimate ascorbic acid as well as *o*-diphenol in colourless reaction mixtures containing the corresponding monophenol, tyrosinase, ascorbic acid and 0.1M phosphate buffer. The reaction was always stopped at the appointed time by pipetting a measured sample into an equal volume of 4% metaphosphoric acid and the protein free filtrate was used for both *o*-diphenol and ascorbic acid estimations. The following account of methods of *o*-diphenol estimation refers, therefore, only to estimations in this particular type of filtrate. The standard solutions of *o*-diphenols, used for the preparation of calibration curves, contained the same amounts of phosphate buffer and metaphosphoric acid as are present in such a filtrate. In no case was the presence of monophenol found to have any effect on the diphenol estimation. Ascorbic acid was only added to the standard solutions when it was found materially to affect the rate of development of the colour which was the basis of the method of estimation.

A 3,4-Dihydroxyphenylalanine (DOPA) Schild (1933) and von Euler (1933) used the red colour developed on oxidation with I_2 for the colorimetric estimation of adrenaline. Evans & Raper (1937*a*) found that a similar method could be used for the estimation of DOPA. The method adopted in the present work was essentially that of Evans & Raper, improved by the use of the Spekker photoelectric absorptiometer instead of visual colour matching. A calibration curve was first prepared. The solution required for establishing a point on the curve was made by mixing in the order given (a) 8.0 ml. of standard DOPA solution,

(b) 14.0 ml. of 0.5M-phosphate buffer (pH 6.0), (c) 10.0 ml. of 0.192N-NaOH and (d) 3.8 ml. of 0.1N- I_2 . This mixture gave a final pH of 6.0. Ninety seconds after the I_2 addition (e) 4.2 ml. of 0.1N $Na_2S_2O_3$ were added, and the mixture immediately transferred to the 4 cm. absorptiometer cell. Absorptiometer readings were taken, with a blue filter (no. 6 of Hilger set H455), at timed intervals for a period of c. 5 min. The red colour remaining in the solution after the $Na_2S_2O_3$ addition, due to the presence of DOPA, was found to diminish appreciably in intensity even within this time. The logarithms of the absorptiometer readings were plotted against time after $Na_2S_2O_3$ addition, and the linearity of the plot permitted extrapolation back to zero time. By following the same procedure with the enzyme reaction filtrate, using the latter instead of (a) above, a zero time absorptiometer reading was obtained and the corresponding DOPA concentration read on the calibration curve. The latter covered DOPA concentrations up to 0.20 mg./ml.

When this method of estimation was applied to samples taken at intervals from an aerated reaction mixture containing initially tyrosinase, ascorbic acid and DOPA (0.2 mg./ml.) but no tyrosine, the DOPA concentrations found 0.5, 5, 10, 15, 20, 30, 40, 50, 60 and 70 min. after the beginning of the enzyme reaction were respectively 0.207, 0.207, 0.206, 0.208, 0.206, 0.207, 0.206, 0.207, 0.204 and 0.208 mg./ml. These figures vouch for the reliability of the method, and for the absence of interference by ascorbic acid, the concentration of which fell from 4.0 to 0.2 mg./ml. during the experiment. They also illustrate the efficiency with which ascorbic acid reconverts DOPA quinone into DOPA.

B Catechol The molybdate method, used by Rae (1930) for adrenaline estimation and said to be applicable to all catechol derivatives, was considered. The statement of Evans & Raper (1937*a*) that ascorbic acid gives a green blue coloration with the molybdate reagent was discouraging, but trial did not reveal any trace of such coloration, and the following procedure was found to be applicable even in the presence of ascorbic acid. For the determination of a point on a calibration curve a solution was made up by mixing (a) 4.0 ml. of catechol standard solution, (b) 7.0 ml. of 0.5M-phosphate buffer (pH 6.0), (c) 5.0 ml. of 0.192N-NaOH and (d) 2.0 ml. of 20% ammonium molybdate. An absorptiometer reading was taken 20 min. after the molybdate addition, using the 1 cm. cell and filter no. 7 of Hilger set H455. The colour intensity continues to increase after its first almost instantaneous development, but not so rapidly (e.g. absorptiometer reading increasing from 0.246 at 20 min. to 0.261 at 150 min.) as to lead to error, provided that the interval between molybdate addition and absorptiometer reading is kept within the limits of 20–30 min. A calibration curve was constructed covering catechol concentrations up to 0.8 mg./ml. To increase the accuracy of the method at the lower concentrations a second curve was prepared using the 4 cm. absorptiometer cells with doubled volumes of solutions (a) to (d) above. This covered a range up to 0.3 mg./ml. For the estimation of catechol formed from phenol in enzymic reaction mixtures, the metaphosphoric acid filtrates of the latter were used in place of solution (a) in the procedure which has been described.

C Homocatechol (4-methylcatechol) When a beginning was made with the preparation of a calibration curve for this estimation by the procedure used for catechol, it was noticed that the solutions prepared for absorptiometer examination,

left standing at room temperature for a day or two, increased in colour intensity by a factor of about 3. The possibility of increasing the sensitivity of the estimation by delaying the colour measurement until this increase had reached its maximum was obvious. Ascorbic acid was found not to affect significantly the colour intensity developing within 20 min. of the addition of molybdate, but it markedly delayed the subsequent increase in colour in such a way as to suggest that the onset of a phase of relatively rapid increase waited upon the exhaustion of the ascorbic acid by spontaneous oxidation. Independently of the amount of ascorbic acid present, the maximum colour finally reached was constant for a given homocatechol concentration and was stable for 2-3 days.

For the preparation of the calibration curves finally used, ascorbic acid was added to the standard homocatechol solutions in an amount equal to the maximum to be expected in the enzyme reaction mixtures (4 mg/ml). The procedure was the same as in the estimation of catechol, but absorptometer readings were taken 20 min. and 3 hr. after molybdate addition and each day until the maximum colour was attained (not more than 5 days). The filter used was Ilford spectrum violet no. 601 which was found to give readings c. 50% greater than the filter previously used. Two calibration curves were drawn, one from the 20 min. readings covering concentrations up to 1.5 mg/ml, the other from the maximum readings for concentrations up to 0.3 mg/ml only. The latter curve was used unless homocatechol concentrations outside its range, giving maximum colours too intense for satisfactory measurement in the absorptometer, were encountered. In the concentration range 0.2-0.3 mg/ml, in which a reliable comparison could be made between assessments based on the 20 min. and on the maximal colour readings, good agreement was found.

D 4.5 Dimethylcatechol. This diphenol behaved very like catechol in the molybdate reaction under the conditions which have been described. For the estimation of 4.5 dimethylcatechol formed enzymically from 4.5 dimethylphenol the procedure was the same as for the homocatechol estimations.

Estimation of ascorbic acid

Samples of the metaphosphoric acid filtrates of the reaction mixtures, which had been prepared as already described for *o*-diphenol estimation, were titrated with a standardized solution of 2.6 dichlorophenolindophenol from a micro burette.

RESULTS

Oxidation of tyrosine

Course of oxidation in presence of ascorbic acid. The results were of the type illustrated in Fig. 1. It will be seen that the observed oxygen uptake was in good agreement with that calculated for the oxidation of ascorbic acid and the formation of DOPA. The lag phase at the beginning of the oxygen-uptake curve corresponds with the lag in the initiation of ascorbic acid oxidation. Since the latter is a secondary oxidation effected by DOPA quinone, as the concentration of DOPA and, therefore, the rate of DOPA-quinone formation increases from zero, the rate of ascorbic acid oxidation also increases. In this experi-

ment it reaches a maximal value when the DOPA concentration has reached 0.0005M, and remains almost linear until all the ascorbic acid has been oxidized. The curve in Fig. 1, showing the changing concentration of DOPA during the experiment, provides no evidence of the occurrence of a lag phase in the oxidation of tyrosine to DOPA.

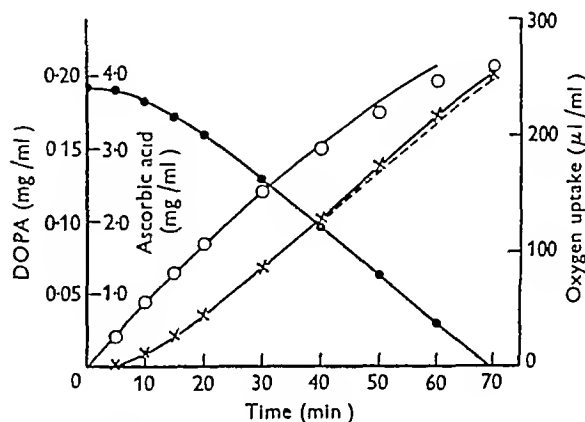


Fig. 1. Oxidation of tyrosine by tyrosinase in the presence of ascorbic acid. Solution saturated with tyrosine, excess solid present. Enzyme concentration, 0.07 mg (dry wt)/ml. ○—○, dihydroxyphenylalanine concentration, the curve representing the expression $Q = 0.77t/(160 + t)$, where Q = concentration and t = time; ●—●, ascorbic acid concentration; x—x, oxygen uptake, the broken line showing the calculated oxygen requirement for ascorbic acid oxidation and DOPA formation.

Decline in rate of formation of DOPA. The rate of DOPA accumulation appears to be maximal at the start, and then progressively declines, although the concentration of DOPA continues to increase so long as ascorbic acid is present to prevent its oxidation beyond the reversible DOPA-quinone stage. Since the concentration of the substrate of the primary oxidation (tyrosine saturated solution with solid phase present) remains constant throughout the experiment, the decline in rate of DOPA formation must be due either to inactivation of enzyme or to inhibition by reaction products, i.e. DOPA or dehydroascorbic acid. In the absence of any other indication of the second of these alternatives, the first seems the more probable, the susceptibility of tyrosinase to inactivation during its reactions being widely recognized.

Calculation of Q_0 for oxidation of DOPA. On the assumption that the decrease in rate of DOPA formation is due solely to enzyme inactivation this rate at any given time, i.e. the slope of the tangent to the DOPA concentration curve at that time, may be taken as a measure of the amount of active enzyme still present. By drawing tangents to the ascorbic acid concentration curve the rate of oxidation of ascorbic acid at any moment may be determined, and this is equal to the rate of oxidation of DOPA to

DOPA quinone From this, by the use of the figure for the amount of residual active enzyme at the same moment obtained in the manner indicated above, one can calculate a Q_{O_2} value for the oxidation of DOPA by the enzyme preparation used in the experiment The Q_{O_2} values so obtained for different times after the beginning of the reaction each relate to a different substrate (DOPA) concentration, the magnitude of which may be found from the DOPA-concentration curve

Thus the data required for the construction of an activity-substrate concentration curve can be abstracted, the activity referred to being the catecholase (as distinct from the monophenolase) activity of the enzyme, with DOPA as substrate This curve

$Q = 0.77t/(160 + t)$, in which Q is the DOPA concentration in mg/ml and t is the time in minutes after the start of oxidation The values of Q calculated from this expression are compared with the observed values in columns 2 and 3 of Table 1 The fourth column of Table 1 gives the rates of DOPA formation at different times, i.e. the calculated values of $dQ/dt = 123/(160 + t)^2$, these being taken as a measure of the amount of enzyme still in the active condition The fifth column shows this amount as a percentage of the amount initially present The Q_{O_2} values for ascorbic acid oxidation, representing DOPA oxidation by the residual active enzyme, are in column 6, and the corresponding DOPA concentrations in molar terms in the last column of the table

Table 1 Oxidation of tyrosine + ascorbic acid by tyrosinase

Time (min)	DOPA concentration (mg/ml)		Rate of DOPA formation (mg/ml/min)†	Enzyme still active (%)	Q_{O_2} of ascorbic acid oxidation	DOPA concentration (10^{-4} M)
	Found	Calc *				
0	0.00	0.00	0.0048	100	—	—
5	0.021	0.023	0.0045	94	—	1.2
10	0.045	0.045	0.0043	90	2400	2.3
15	0.065	0.066	0.0040	83	3000	3.3
20	0.085	0.086	0.0038	79	3700	4.3
25	—	0.104	0.0036	75	4500	5.2
30	0.120	0.122	0.0034	71	5000	6.2
40	0.150	0.154	0.0031	66	5600	7.8
50	0.175	0.183	0.0028	58	6150	9.3
60	0.197	0.210	0.0025	52	6800	10.7

* Calculated from $Q = 0.77t/(160 + t)$
† Calculated from $dQ/dt = 123/(160 + t)^2$

may then be compared with the corresponding curve determined in direct experiments with the enzyme and DOPA, if the two curves coincide, the above interpretation of events in the reacting system would be strongly supported

Rate of DOPA formation at zero time In attempting to treat the experimental data in the above way, a primary difficulty is in the satisfactory assessment of the rate of DOPA formation at zero time, before any enzyme inactivation has taken place For the oxidation of catechol by tyrosinase in the presence of ascorbic acid Miller & Dawson (1941) have shown that the experimental data fit the relationship $Q = at/(b + t)$, where Q is the total substrate which has been oxidized in time t , and a and b are constants, the magnitude of b being determined by the rate of inactivation of the enzyme The reciprocal of Q plotted against the reciprocal of t gives a straight line, and as the slope of this line is the reciprocal of the initial reaction velocity (dQ/dt for $t = 0$) the latter may readily be determined with considerable accuracy

Applying this device in the instance under discussion, it is found that the DOPA-concentration curve during the greater part of the experiment may be represented reasonably well by the expression

Reaction velocity and DOPA concentration In a separate series of manometric experiments the relationship between reaction velocity and substrate

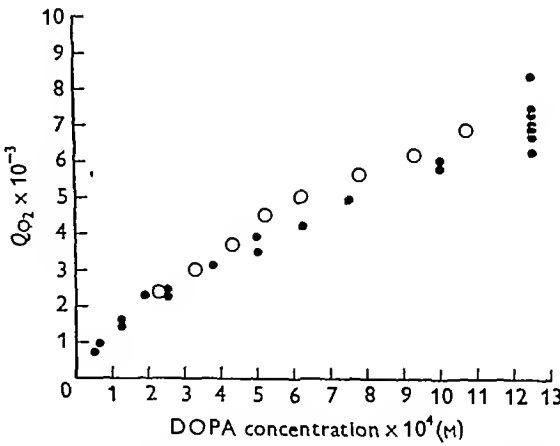


Fig 2 Oxidation of DOPA by tyrosinase Enzyme activity-substrate concentration curve ●, direct determinations, ○, calculated from data of tyrosine oxidation experiment

(DOPA) concentration was studied directly DOPA itself in known concentration without tyrosine was used as substrate, and ascorbic acid and buffer concentrations were the same as in the experiments

already described. The oxygen uptake during the first 5 min after the addition of the enzyme to the DOPA-ascorbic-buffer mixture was used for the purpose of calculating the Q_{O_2} value. The Q_{O_2} values obtained by this direct method are plotted against substrate concentration in Fig 2, together with the values of Table 1 calculated from the data of the tyrosine oxidation experiment. The two sets of values are seen to be consistent with each other, and this lends support to the interpretation of the tyrosine oxidation experiment which has been put forward.

rate continued to increase until in saturated (c 0.025M) DOPA solution Q_{O_2} values of about 25,000 were recorded.

Q_{O_2} of true monophenolase and of the overall reaction. The initial rate of DOPA formation in the experiment represented in Fig 1, calculated from the reciprocal plot of the DOPA accumulation curve, was 0.0048 mg/ml/min, which corresponds to a Q_{O_2} (μ l O_2 /mg dry wt/hr) of 230 for the enzyme preparation used. When tyrosine in saturated solution in 0.1M-phosphate buffer (pH 7.0) was oxidized in the absence of ascorbic acid, with the same enzyme

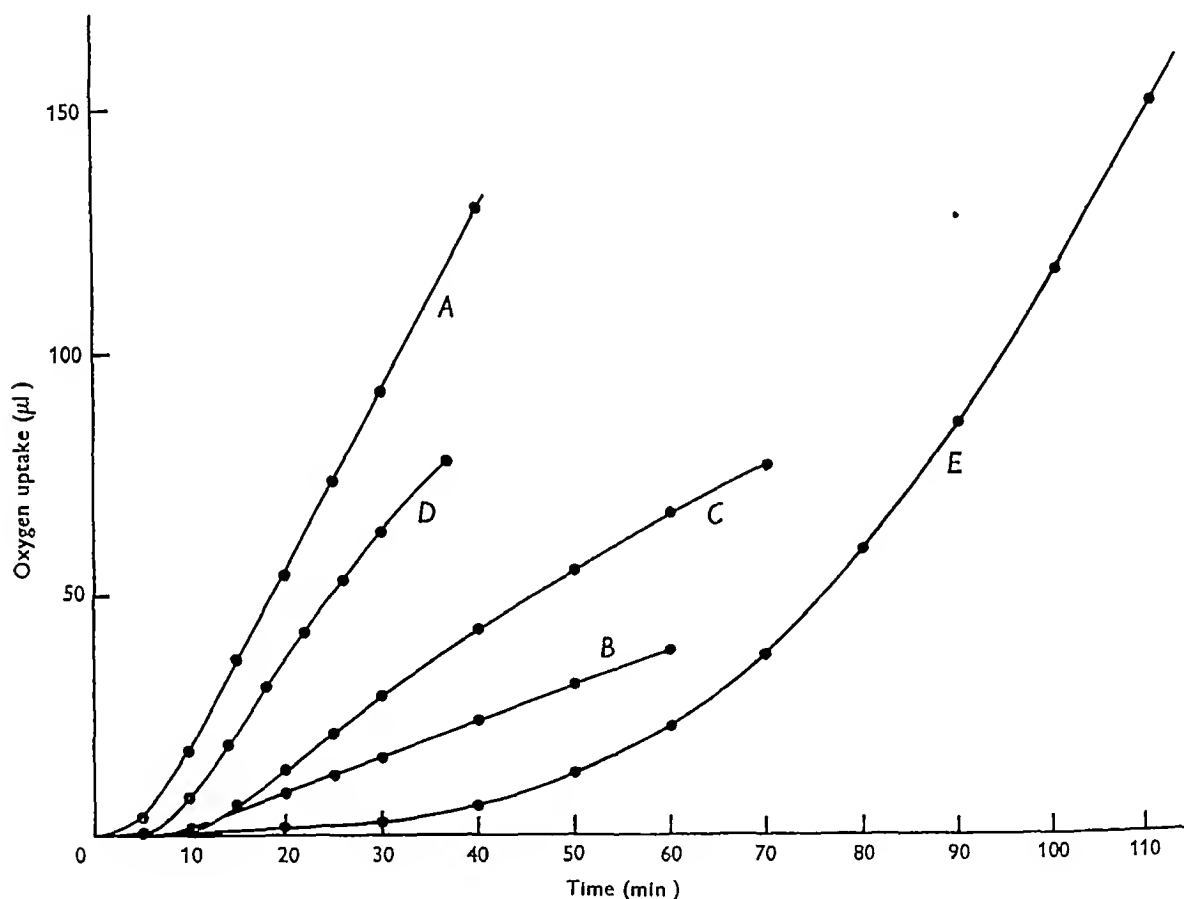


Fig 3. Oxidation of monophenols with tyrosinase, in the absence of ascorbic acid. Curves A and B, tyrosine, saturated solution (c 0.0025M), A, 0.35 mg enzyme, B, 0.07 mg enzyme. Curve C, 0.005M phenol, 0.02 mg enzyme. Curve D, 0.005M *p*-cresol, 0.007 mg enzyme. Curve E, 0.017M 4,5-dimethylphenol, 0.35 mg enzyme.

It follows from this result that the presence of tyrosine in saturated solution (c 0.0025M) does not significantly inhibit the oxidation of DOPA by the enzyme. The apparently linear course of the oxidation of ascorbic acid seen in Fig 1 after the maximal rate is reached must be ascribed to a fortuitous balance of two opposing factors, viz. the progressive inactivation of the enzyme which would of itself diminish, and the progressive increase in DOPA concentration which would of itself increase, the reaction rate. With continued increase in DOPA concentration above the range of Fig 2 the reaction

preparation, the oxygen uptake followed the curves shown in Fig 3. After the induction period the rate of uptake became nearly linear at values of 3.82 and 0.75 μ l/min at the two enzyme concentrations employed, one of which was five times the other. The corresponding Q_{O_2} values are 650 and 640, and two other similar determinations gave values of 650.

These Q_{O_2} values of 230 for the true monophenolase activity and 650 for the overall reaction are in accordance with the relative speeds of the component steps in the oxidation of tyrosine suggested by Evans & Raper (1937b). According to the scheme put

forward by these authors the primary slow oxidation to DOPA, requiring one atom of oxygen/mol of tyrosine, is followed by three successive relatively fast reactions, two of which, the conversion of DOPA to DOPA quinone, and, after ring closure, the oxidation of 5,6 dihydroxydihydroindole-1 carboxylic acid to its quinone, each require one atom of oxygen. If the secondary reactions are markedly faster than the first reaction, one would then expect the Q_{O_2} for the whole oxidation to be approximately three times the Q_{O_2} of the isolated primary reaction. The activity substrate concentration curve of Fig 2 shows that the Q_{O_2} for DOPA oxidation reaches a value of 230 at a DOPA concentration of c 0.0002M. During the oxidation of tyrosine by tyrosinase in the absence of ascorbic acid one would, therefore, not expect DOPA to accumulate beyond this concentration, at which rate of removal would balance rate of formation, and the difficulty experienced by earlier workers (Raper, 1926) in attempting to demonstrate DOPA formation by isolation is consequently not surprising.

Oxidation of phenol

Rate of formation of catechol in presence of ascorbic acid The results obtained with phenol were, except in one respect, qualitatively similar to the results of the tyrosine experiments. The course of catechol accumulation at the three phenol concentrations studied is shown in Fig 4. The curves provide no evidence of an induction period in the primary oxidation of phenol to catechol. The catechol concentrations which had been built up when the experiments were terminated by the exhaustion of the ascorbic acid were 0.0053M from 0.1M-phenol, 0.0021M from 0.01M phenol and 0.00095M from 0.001M phenol, representing a conversion of 5, 21 and 95% respectively of the substrate initially present. Only in the case of the lowest phenol concentration is the fall in primary substrate concentration during the course of the experiment likely to have been a significant factor in bringing about the slowing down of catechol formation, and the form of the catechol accumulation curves at the higher phenol concentrations is attributed to the result of enzyme inactivation. These curves can be represented reasonably well by the equations $Q = 2.0t/(122 + t)$ and $Q = 0.53t/(38 + t)$, the lines drawn in Fig 4 are the plots of these equations and show the closeness of fit.

The equations give initial Q_{O_2} values for the isolated primary oxidation of 2900 with 0.1M- and 2400 with 0.01M-phenol and in other similar experiments figures of 3100 and 2700, respectively, were obtained. In the experiment with 0.001M-phenol the initial Q_{O_2} relating to this concentration cannot be judged with confidence because of the rapid fall in substrate concentration. The use of the first two points only of

the catechol accumulation curve by the reciprocal plot method gives a Q_{O_2} of 1900. When phenol was presented as substrate to the same enzyme preparation in the absence of ascorbic acid, the initial lag in oxygen absorption was much more pronounced and the uptake followed a course such as that shown in Fig 3. The maximal rate of oxygen usage, reached after 20 min, corresponds to a Q_{O_2} of 4700, which is of the order of magnitude to be expected if the rate of the primary reaction determines the overall oxygen uptake rate.

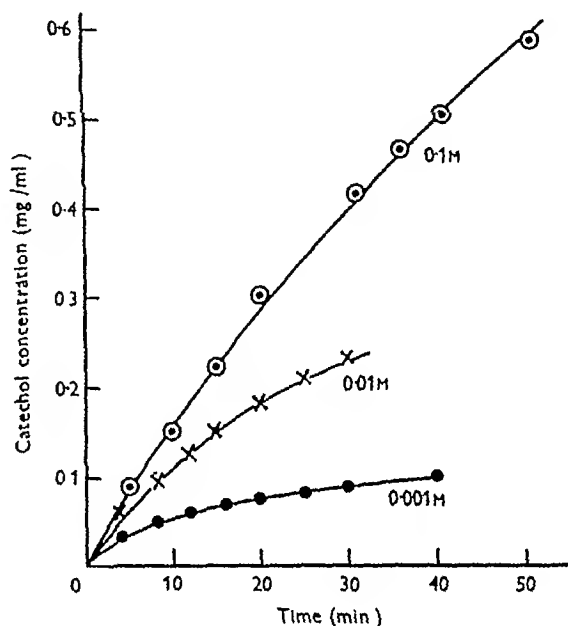


Fig 4 Accumulation of catechol during the oxidation of phenol by tyrosinase in the presence of ascorbic acid. The initial phenol concentration is indicated alongside each curve. Enzyme concentration, 0.035 mg/ml. The curve drawn for 0.1M phenol represents the expression $Q = 2.0t/(122 + t)$, and that for 0.01M phenol the expression $Q = 0.53t/(38 + t)$, Q being catechol concentration in mg/ml.

Inhibition of catechol oxidation by phenol The results obtained in the experiments with phenol differed qualitatively from those given by tyrosine in one respect. Analysis of the results with tyrosine produced no evidence that tyrosine had any inhibitory effect on the oxidation of DOPA by the enzyme. The results of a similar analysis of the data of the phenol experiments, however, could only be satisfactorily interpreted if an inhibitory effect on catechol oxidation were attributed to phenol. It is not proposed to give this analysis in detail, since the same effect is seen in a more intense form during the oxidation of 4,5 dimethylphenol, to be described later. The inhibitory effect of phenol on catechol oxidation was demonstrated in direct experiments such as that illustrated in Fig 5. The inhibition was competitive in type. When catechol and phenol were present in equimolar proportions the phenol was

without significant effect. But when the relative catechol concentration was reduced to $1/5$ there was marked inhibition, and at $1/20$ strong inhibition, of the initial oxygen-uptake rate. It must be borne in mind that in the phenol-containing reaction mixtures the concentration of catechol increases from the moment at which the enzyme is added, owing to the conversion of phenol to catechol by the enzyme. As a result of the increase in catechol concentration and the decrease in phenol concentration, the inhibition might be expected to pass off with time, and that it does so is evident in the curves.

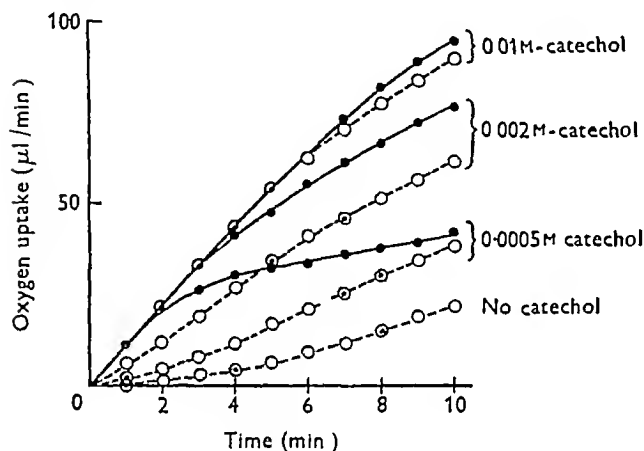


Fig 5 Effect of phenol on oxidation in the ascorbic acid + catechol + tyrosinase system. ●—●—●, without phenol, ○—○—○, with 0.01M phenol

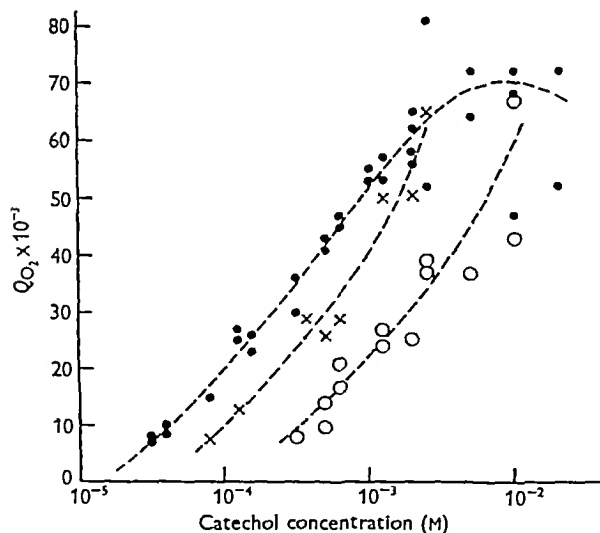


Fig 6 Oxidation of catechol by tyrosinase. Effect of phenol on the enzyme activity substrate concentration curve. Dots, no phenol present, crosses, with 0.001M phenol, circles, with 0.01M-phenol

Q_{O_2} of catechol oxidation. The Q_{O_2} values obtained in a series of determinations similar to those shown in Fig 5 and using reaction mixtures with various catechol concentrations and (a) no phenol, (b) 0.001M-

phenol and (c) 0.01M-phenol are plotted against catechol concentration in Fig 6. The Q_{O_2} values are based on the oxygen uptake in the first 2 min of the reaction and are admittedly subject to a rather large possibility of experimental error, and this is evident in the scatter of points in Fig 6. But the nature of the relationship between enzyme activity and catechol concentration, and the competitive inhibitory effect of phenol, are clearly discernible. One half the maximal activity is reached at c 0.0003M-catechol in the absence of phenol and at c 0.0007 and 0.002M-catechol in the presence of 0.001 and 0.01M phenol respectively.

Oxidation of *p* cresol

Course of homocatechol formation in presence of ascorbic acid. The course of homocatechol accumulation in a series of experiments at different *p* cresol

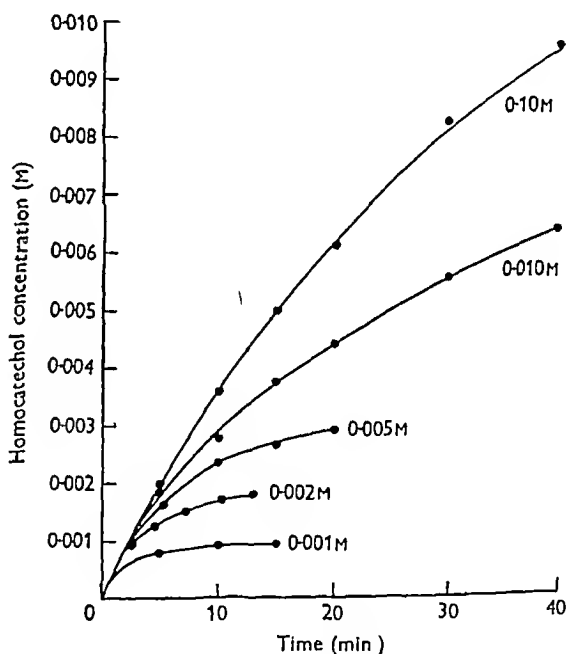


Fig 7 Accumulation of homocatechol during oxidation of *p* cresol with tyrosinase in the presence of ascorbic acid, enzyme concentration, 0.035 mg (dry wt)/ml. The initial *p* cresol concentrations in the various experiments are recorded at the ends of the curves. The uppermost curve represents the expression $Q = 2.5t/(45.6 + t)$

concentrations is shown in Fig 7. The enzyme concentration was the same as in the experiments with phenol, but the formation of *o* diphenol was much faster. With the smaller *p* cresol concentrations, the asymptotic approach to complete conversion to homocatechol is apparent and even with 0.01M-*p*-cresol 64% conversion had taken place in 40 min. Only at the highest *p*-cresol concentration (0.1M) is the resultant decrease in *p* cresol sufficiently small relative to the initial concentration to make it

unlikely that the rate of the primary oxidation is affected during the course of the experiment by the fall in substrate concentration

The uppermost curve in Fig 7 is the curve of the expression $Q = 2.5t/(45.6 + t)$, where Q is the homocatechol concentration in mg/ml. This expression fits the points for the experiment with 0.10M-*p*-cresol very well. The initial reaction velocity ($t=0$) is 0.55 mg/ml/min corresponding to a Q_{O_2} of 8500. Using the homocatechol formation in the first 5 min for the calculation of Q_{O_2} values to represent the monophenolase activity of the enzyme in each case, the results shown in Table 2 were obtained. The *p*-cresol concentrations are the means of the values at the beginning and at the end of the 5 min period. The figures suggest that half the maximal reaction velocity is reached at a substrate concentration of about 0.001M.

Table 2 Oxidation of *p*-cresol + ascorbic acid by tyrosinase monophenolase activity at various substrate concentrations

<i>p</i> -Cresol concentration (M)	Monophenolase Q_{O_2}
0.098	7880
0.0096	7200
0.0046	6200
0.0013	5180
0.0006	3100

Q_{O_2} of overall oxidation. When *p*-cresol in the absence of ascorbic acid is oxidized by excess of tyrosinase, the oxygen uptake approaches three atoms/molecule of *p*-cresol. If the overall rate of the reaction is determined by the rate of the primary oxidation of *p*-cresol to homocatechol, one would expect it, in terms of oxygen-uptake rate, to be about three times that of the primary oxidation. The course of oxygen uptake when the enzyme preparation used in this series of experiments oxidized *p*-cresol in the absence of ascorbic acid is shown in Fig 3. The maximal rate, reached after 10 min, corresponds to a Q_{O_2} of 25,000, which is of the order of magnitude expected in relation to the figure of about 8500 found for the isolated primary reaction.

Q_{O_2} of homocatechol oxidation. Inhibition by monophenol. Using the rate of ascorbic acid oxidation in these experiments as an index of the rate of homocatechol oxidation, Q_{O_2} values were calculated in the manner already given in the description of the tyrosine experiments. They were found to be lower than the values obtained in direct determinations with homocatechol as substrate at equivalent concentrations. As in the case of phenol-catechol, this suggested the operation of an inhibitory effect of the monophenol on the oxidation of the corresponding o-diphenol, which was verified by direct experiment (Fig 8).

Oxidation of 4,5-dimethylphenol + ascorbic acid

Rate of accumulation of 4,5-dimethylcatechol. This was found by the reciprocal plot method to conform well with the relationships $Q = 0.76t/(49 + t)$ and $Q = 1.0t/(115 + t)$ for initial monol concentrations of

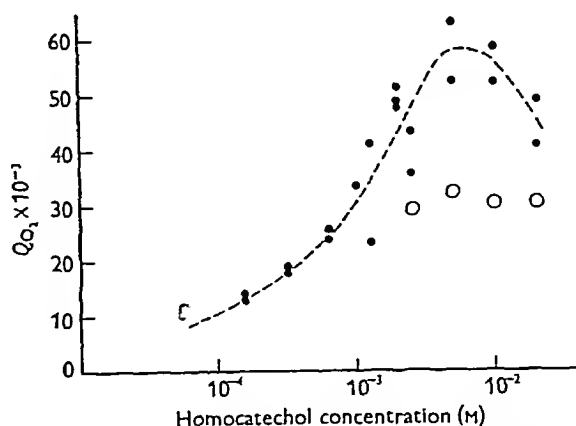


Fig 8 Oxidation of homocatechol by tyrosinase. Enzyme activity-substrate concentration curve O O, Q_{O_2} values determined in the presence of 0.01M-*p*-cresol

0.02 and 0.005M respectively, Q being the 4,5-dimethylcatechol concentration in mg/ml/min after time t . The initial rates of diol formation in the two cases ($dQ/dt, t=0$) correspond to Q_{O_2} values of 330 and 180. No attempt was made to treat the data obtained at lower initial monol concentrations in this way, the method of estimation of the diol is not sufficiently reliable at lower concentrations, and there is the additional complication of a relatively rapidly changing monol concentration. At 0.001M, the latter had been reduced to half in about 20 min.

Q_{O_2} for diphenol oxidation in presence and absence of the monophenol. In the experiments at the lower 4,5-dimethylphenol concentrations, the lag in the initiation of ascorbic acid oxidation and oxygen absorption was much more prolonged, and the subsequent acceleration much more dramatic, than in any of the experiments with the other substrates. The general nature of the difference may be appreciated if Fig 9, showing the results of an experiment with 0.001M-4,5-dimethylphenol, is compared with Fig 1. The results of an analysis of the data of Fig 9 are recorded in Table 3. The rates of ascorbic acid oxidation given in the table were obtained from the slopes of tangents drawn to the ascorbic acid concentration curve at the appropriate points. They are taken to be equivalent to the rates of oxidation of 4,5-dimethylcatechol and the Q_{O_2} (catecholase) values given in the third column of the table are based on them. The dimethylcatechol concentrations in column 4 are taken from the accumulative curve of Fig 9.

The rate of oxidation of ascorbic acid during the first 20 min of this experiment was so small as to be within the range of spontaneous oxidation under these conditions in controls without enzyme, so that even the very small Q_{O_2} values recorded in this period must have been seriously overestimated. The later Q_{O_2} values, on the other hand, would be subject to a progressively greater magnification if it were possible to relate them to the amount of residual

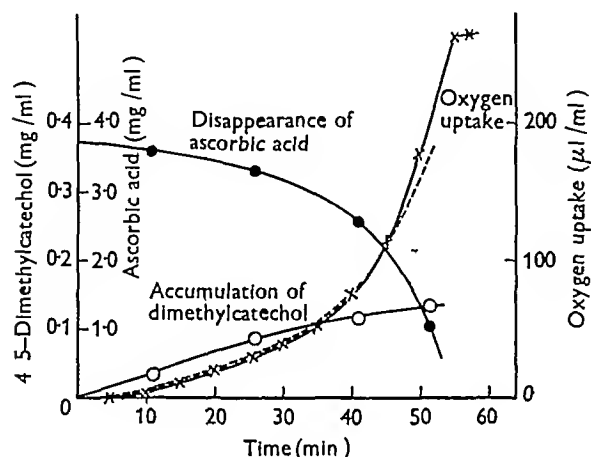


Fig 9 Oxidation of 0.001M 4,5 dimethylphenol by tyrosinase in the presence of ascorbic acid. Enzyme concentration, 0.115 mg (dry wt)/ml. The broken line shows the calculated oxygen requirement for ascorbic acid oxidation and dimethylcatechol formation.

active enzyme instead of to the amount of enzyme initially present. These considerations together indicate that the real increase in Q_{O_2} (catecholase) must have had a more sudden onset than the figures in Table 3 suggest. Since, in the absence of 4,5-dimethylphenol, Q_{O_2} values for the oxidation of 4,5-dimethylcatechol were found to be of the order of 40,000 at a dimethylcatechol concentration such as had been reached within less than 10 min of the beginning of the experiment under discussion (Fig 10), the delay in the attainment of a high Q_{O_2} must be due to the inhibitory effect of the dimethylphenol present.

The acceleration of ascorbic acid (=dimethylcatechol) oxidation is attributed to (a) the accumulation of the substrate 4,5 dimethylcatechol and

(b) the removal of the inhibitory effect of the monol on the oxidation of the diol, as the relative concentration of these changes. It is most marked when the conversion of monol to diol is approaching completion and the monol/diol ratio changing rapidly to

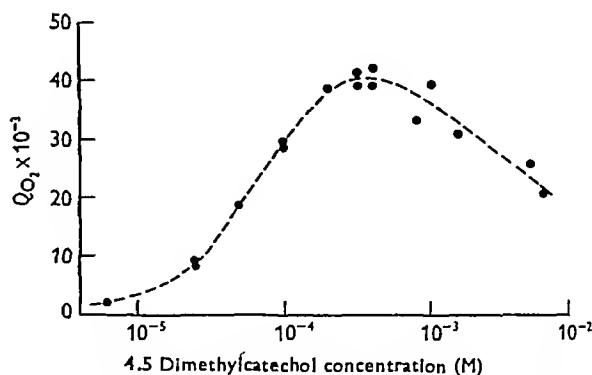


Fig 10 Oxidation of 4,5 dimethylcatechol by tyrosinase, enzyme activity substrate concentration curve.

very small values. The change in this ratio is shown in the last column of table 3. At higher initial dimethylphenol concentrations, e.g. 0.02M, the molar ratio monol/diol remained greater than unity and the inhibitory effect of monol on the diol oxidation was maintained throughout the experiment.

The course of oxygen uptake in manometric experiments in which smaller concentrations of 4,5-dimethylphenol were used is seen in Fig 11. In those curves which relate to primary substrate concentrations of less than 0.002M the very slow initial uptake and subsequent rapid acceleration are strikingly evident. The initial rate is scarcely measurable, and of an order of magnitude accountable in terms of the monophenolase activity of the enzyme and the autooxidation of ascorbic acid. For a given enzyme concentration, the lower the concentration of dimethylphenol the earlier and the more sudden is the onset of the rapid phase of oxygen absorption, maximal rates with 0.0004, 0.0001 and 0.000025M dimethylphenol are reached respectively in 80, 22 and 8 min (Fig 11b). On the other hand, at a given substrate concentration the rapid phase appears earlier, the greater the enzyme concentration, with 0.0004M-dimethylphenol, at 10-15 min.

Table 3. Oxidation of 4,5-dimethylphenol + ascorbic acid by tyrosinase
(Dimethylphenol concentration initially 0.001M)

Time (min)	Rate of ascorbic acid oxidation (μl. O ₂ /ml/min)	Q_{O_2} * catecholase	Dimethylcatechol concentration (10 ⁻³ M)	Dimethylphenol concentration (10 ⁻³ M)	Molar ratio monol/diol
10	0.8	610	0.26	0.74	2.9
20	1.3	670	0.49	0.51	1.0
30	2.3	1200	0.69	0.31	0.45
40	5.1	2600	0.84	0.16	0.19
50	12.4	6400	0.94	0.06	0.06

* Not corrected for inactivation

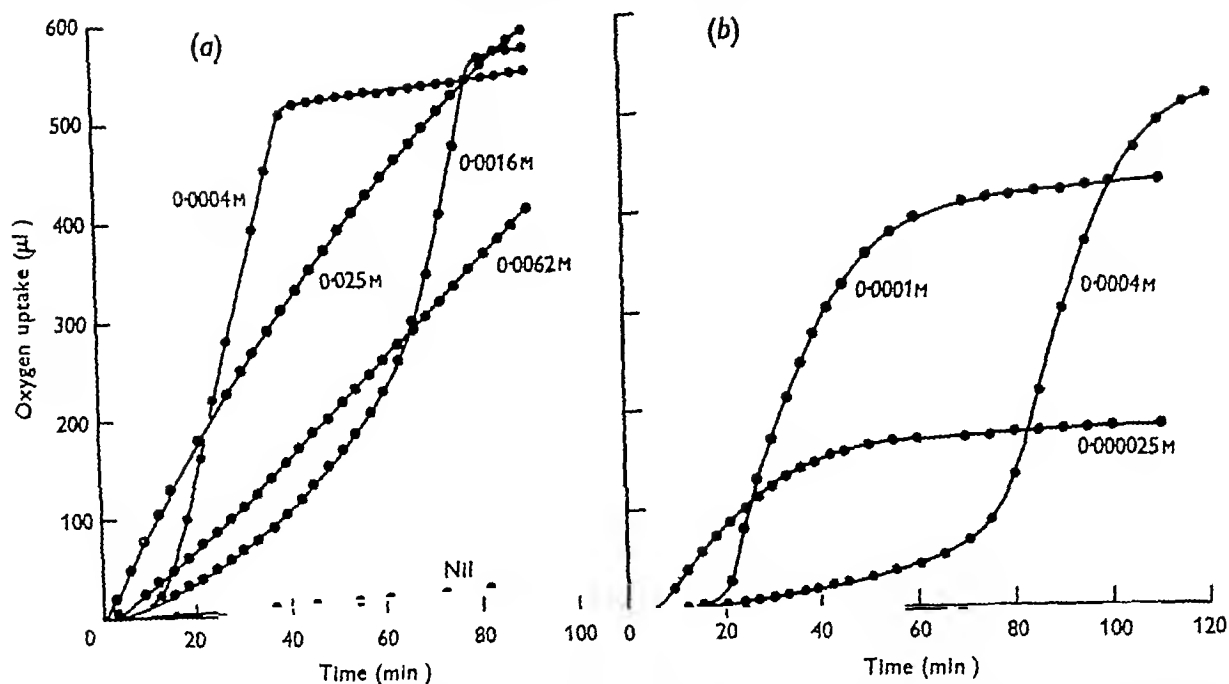


Fig 11 Oxidation of 4,5 dimethylphenol + ascorbic acid by tyrosinase. Reaction mixture vol 2.0 ml, containing 8 mg ascorbic acid ($\equiv 508 \mu\text{l O}_2$). Initial dimethylphenol concentrations as stated against the curves, (a) 0.23 mg, (b) 0.058 mg, tyrosinase (dry wt)

with 0.232 mg enzyme (Fig 11a), but at 70–80 min with 0.058 mg enzyme (Fig 11b). This is in agreement with the interpretation already put forward, that the catecholase function of the enzyme is strongly inhibited by the monophenol, the inhibition passing off swiftly when the ratio monophenol/diphenol falls rapidly as the conversion of the one to the other approaches completion. At the higher dimethylphenol concentrations, 0.025 and 0.0062M (Fig 11a), the rapid phase of oxygen uptake was not reached during the course of the experiment, the earlier experiments in which dimethylcatechol concentrations were estimated indicated that this is because the concentration of dimethylphenol remained sufficiently high in these cases to exert a strong inhibitory effect throughout the experiments. At the lowest dimethylphenol concentrations (less than 0.0004M) the rapid phase of oxygen uptake finishes before all the ascorbic acid has been oxidized (which requires $508 \mu\text{l O}_2$), it thus appears that in this concentration range the rate of enzyme inactivation becomes greater with diminishing substrate concentration.

Inhibition of diphenol oxidation by monophenol The inhibitory effect of 4,5-dimethylphenol on the oxidation of 4,5-dimethylcatechol was demonstrated directly in systems containing ascorbic acid, enzyme and known concentrations of the two substrates. The results of a typical experiment are shown in Fig 12, where the course of oxygen uptake in the first few minutes in systems containing 10^{-3}M -dimethylcatechol and various concentrations of di-

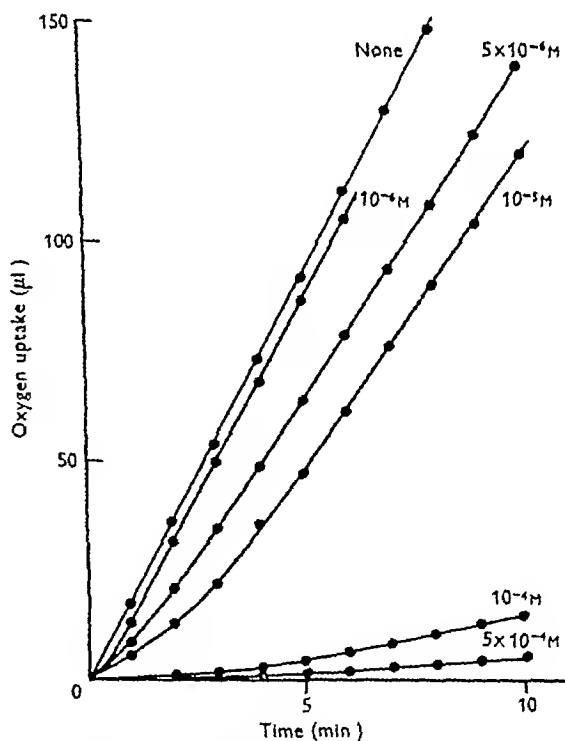


Fig 12 Effect of 4,5 dimethylphenol on oxidation in the 4,5 dimethylcatechol + ascorbic acid + tyrosinase system, 0.029 mg enzyme (dry wt), 4,5 dimethylcatechol concentration, 10^{-3}M , 4,5 dimethylphenol concentrations as stated against each curve

methylphenol are plotted. There is a marked depression of the initial uptake rate with $5 \times 10^{-4}\text{M}$ -monol, i.e. when the ratio of monol to diol is only

1 200, and the inhibition is practically complete when this ratio is 1 10. At the lower monol concentrations the inhibition quickly passes off, as may be expected owing to the conversion of monol to diol by the enzyme. A rough quantitative estimate of the extent of the inhibition in this and another similar experiment is given in Table 4. The estimate is based on the oxygen uptake in the first minute, but it should be remembered that with the very low monol concentrations the inhibition may already be passing off even within this short time, on account of diminishing monol concentration.

Table 4 *Inhibition of oxidation of 4 5 di-methylcatechol by 4 5 dimethylphenol*

Initial concentration (10 ⁻³ M)		Ratio monol/diol	Initial O ₂ uptake rate (μl/min)	Inhibition (%)
Diol	Monol			
1 0	Nil	—	18 0	—
	0 001	0 001	13 5	25
	0 005	0 005	9	50
	0 01	0 01	6 5	64
	0 1	0 1	1	94
	0 5	0 5	0 5	100
0 3	Nil	—	20	—
	0 0012	0 004	8 5	57
	0 005	0 016	5	75
	0 02	0 07	1 5	93

Oxidation of 4 5 dimethylphenol in absence of ascorbic acid. In this case, the induction period before maximal oxygen-uptake rate was attained was much longer than in the cases of the other monophenolic substrates under the same conditions. The oxygen uptake in such an experiment is included in Fig. 3. The maximal rate corresponded to a Q_{O_2} of 620, reached after the reaction had proceeded 100 min. This may be compared with the value of 330 found for the isolated primary reaction in the presence of ascorbic acid at a similar dimethylphenol concentration.

DISCUSSION

The behaviour of the reacting system containing monophenol, tyrosinase and ascorbic acid can in each of the cases studied be adequately explained in terms of (a) specific monophenolase activity of the enzyme preparation, maximally effective, in the presence of ascorbic acid, at the moment of addition of enzyme to substrate, (b) specific catecholase activity of the preparation, (c) progressive inactivation of the enzyme during the reaction, and (d) competitive inhibition of catecholase activity by monophenols. The relative magnitude of these factors determines the course of oxidation in each particular case. Comparing the oxidation of tyrosine + ascorbic acid with that of 4 5 dimethylphenol + ascorbic acid, for example, we find that the Q_{O_2} values for the isolated primary oxidations were

not very different (230 for 0 0025M-tyrosine, 180 for 0 005M-dimethylphenol). The oxygen uptake reached its maximal rate much more rapidly with tyrosine than with dimethylphenol, because dimethylphenol strongly inhibits the oxidation of the dimethylcatechol formed from it in the primary reaction, whereas tyrosine has no such inhibitory effect on DOPA oxidation. Again, the effect of this inhibition in accentuating and prolonging the induction phase of the oxygen-uptake curves is much more striking in the case of dimethylphenol than with phenol or *p*-cresol not only because the inhibition for a given monol/diol concentration ratio is more powerful, but also because the rate of diphenol formation is much slower, the relative monophenolase activities for the substrates phenol/*p*-cresol/dimethylphenol being 1 0/2 6/0 1, whereas the relative catecholase activities with catechol/homocatechol/dimethylcatechol were 1 00/0 83/0 59.

Reference was made in the introduction to the conclusion of other workers that the oxidation of monophenols by tyrosinase is dependent in some way upon the simultaneous oxidation of *o*-diphenol by the enzyme, though not a result of secondary oxidation of monophenol by products of oxidation of diphenol. In the case of each monophenol studied in the present work, however, the rate of the primary oxidation to *o*-diphenol appears to be maximal at the very beginning of the enzyme reaction, and, when an induction period is evident before the maximal oxygen-uptake rate is reached, it is due in the main to the inhibiting effect of monophenol on diphenol oxidation. This leads inevitably to the conclusion that the presence of *o*-diphenol is not in fact necessary for monophenolase activity to be displayed. How then is the induction period observed when monophenol is oxidized in the absence of ascorbic acid to be explained? With 4 5 dimethylphenol as substrate the Q_{O_2} of the primary reaction in the presence of ascorbic acid was found to be of the order of 300 for the enzyme used in this work. In the absence of ascorbic acid, with the same substrate, the total oxygen-uptake rate reached a value corresponding to $Q_{O_2} = 620$, but only after a long induction period during the earlier part of which the oxygen uptake rate was almost zero (Fig. 3). This maximal value of 620 does not conflict with a Q_{O_2} (monophenolase) of about 300, but the fact that the initial oxygen-uptake rate was very much smaller than that appropriate to a Q_{O_2} of 300 points to the conclusion that in the absence of ascorbic acid the monophenolase function of the enzyme was at first relatively quiescent, and that it was activated by *o*-diphenol as the latter slowly accumulated. Ascorbic acid appears to bring about an almost instantaneous activation of monophenolase function and its effect in this respect is not, as other workers have supposed (Nelson & Dawson, 1944), dependent on its favouring *o*-diphenol accu-

mulation. Indeed from the moment of completion of the reaction mixture by addition of enzyme onwards, monophenolase activity diminishes on account of enzyme inactivation, in spite of the progressive increase of *o*-diphenol concentration and oxidation. Ascorbic acid and *o*-diphenols have in common rather strong reducing power and similar reducing groups, and it seems possible that the effect which they have on monophenolase function may be dependent on this property. At any rate the new evidence is against the existence of any specific role for *o*-diphenols in relation to monophenol oxidation.

It is regrettable that the methods available for *o*-diphenol estimation did not permit the studies of *o*-diphenol formation to be carried out over a sufficient range of monophenol concentration to provide reliable activity substrate concentration curves for the isolated primary oxidation. With each monophenol substrate, monophenolase activity was highest at the highest substrate concentration employed, even when this was as great as 0.1M, and there was no sign of inhibition such as is observed with the *o*-diphenolic substrates at such high concentrations. The strong competitive inhibitory effect of quite low concentrations of 4.5 dimethylphenol on the catecholase function of the enzyme suggests a much greater affinity of the specific 'catecholase centre' in the enzyme for this monophenol than for the *o*-diphenols or other monophenols used. Were the same centre responsible for monophenol oxidation, one would expect the substrate concentration necessary to saturate the enzyme to be particularly low in the case of dimethylphenol. The few facts available are not in accordance with this expectation. In the case of dimethylphenol the Q_{O_2} (monophenolase) was 330 at 0.02M substrate, but only 180 at 0.005M-substrate, whilst with *p*-cresol a value of 7200 at 0.01M had fallen only to 5000 at 0.001M, suggesting that the affinity for dimethylphenol was appreciably less than for *p*-cresol. Thus it appears probable that the activities of the enzyme or enzyme complex in respect of monophenol and *o*-diphenol are located at different centres.

The optimal concentrations of the *o*-diphenolic substrates were 0.004M for catechol, 0.006M for homocatechol and 0.00035M for 4.5 dimethylcatechol. Two of these values agree reasonably well with the figures given by Nelson & Dawson (1944), 0.002 and 0.0007M for catechol and dimethylcatechol respectively. For homocatechol these authors give 0.0006M as the optimal concentration and there appears to be no obvious explanation of the discrepancy. It would be unwise to assume that these differences in optimal concentration reflect differences in enzyme substrate affinities, in the absence of knowledge of other factors which determine the shapes of the activity substrate concentration curves, and in particular of the reason for

the falling off in activity at high substrate concentrations, which is especially marked in the case of dimethylcatechol. The relative activity towards the three *o*-diphenolic substrates, at the optimal concentration of each, of 1.00 : 0.83 : 0.59, does not differ greatly from the ratio of 1.00 : 0.88 : 0.74 quoted by Nelson & Dawson (1944).

So far as the experiments which have been described show, the monophenolase and catecholase activities of tyrosinase appear to be functionally independent, except as regards the inactivation of the enzyme during the reaction. In the treatment of the data obtained in the monophenol oxidation experiments, the rate of formation of *o*-diphenol was taken as a measure of the amount of enzyme still in the active condition, in those experiments in which it was considered that the decline in this rate could not be attributed to the diminution in monophenol concentration. Catecholase activity, assessed in terms of oxidation rate of ascorbic acid, was then related to the amount of residual active enzyme determined in this way. The Q_{O_2} (DOPA) values thus calculated from the data of the tyrosine oxidation experiment were found to agree very well with those obtained by direct manometric studies of the oxidation of DOPA itself (Fig. 2), and this appears to justify the calculation, but in the calculation the amount of residual active enzyme, to which the DOPA oxidation was attributed, was judged by the extent of residual monophenolase activity. This suggests that the process of inactivation affects equally both the catecholase and monophenolase functions, and, therefore, that these are properties of one and the same enzyme complex. The extent to which the data relating to the oxidation of the other monophenols conform with this view is not readily judged, because of the additional complicating factor of inhibition of *o*-diphenol oxidation by the monophenol present.

Nearly all previous experimental work on the oxidation of monophenols by tyrosinase has been carried out with the initially simpler system containing only monophenol, enzyme and buffer. The quantitative study of *o*-diphenol formation in such systems presents obvious difficulties on account of the pigment developed, but it is hoped that such studies may soon be made. It is by no means certain, however, that the behaviour of the enzyme in the absence of ascorbic acid is of greater biological significance than its behaviour in the presence of this substance. Of the two sources of tyrosinase which have been chiefly used, the mushroom is stated to contain no ascorbic acid (Medical Research Council, 1945), but the potato may contain up to 30 mg/100 g. Several authors have suggested that the *o*-diphenol-tyrosinase system may play a part in the economy of plant tissues, by forming the terminal link in a respiratory chain (Boswell & Whiting, 1938,

Baker & Nelson, 1943) According to their view the *o*-quinone formed in the presence of oxygen by the enzyme abstracts hydrogen from some unspecified hydrogen donator and is consequently reconverted to *o*-diphenol. In the potato one would expect ascorbic acid to be a powerful competitor for this quinone, unless it is in some way separated spatially from the enzyme in the tissue cells. The monophenolase function appears to have been regarded by the proponents of this theory merely as a mechanism for providing the *o*-diphenol necessary for such a respiratory function. Robinson & Nelson (1944) formed the opinion that in the presence of tyrosinase only a trace of DOPA sufficient for the above purpose accumulated, and suggested, to explain this, that the oxidation of tyrosine was strongly inhibited by DOPA. No direct experimental evidence in support of these speculations was given, however, and the evidence now presented has shown that considerably more than traces of DOPA may accumulate. There was, it is true, a falling off in the rate of DOPA formation during the enzyme reaction, although the tyrosine concentration remained constant, but this can be adequately accounted for by enzyme inactivation. The possibility that the monophenolase function of tyrosinase may also be important for syntheses of substances other than the substrate for the catecholase function ought still to be borne in mind.

SUMMARY

1 Colorimetric methods for the estimation of *o*-diphenol in enzymic reaction mixtures containing

ascorbic acid and the corresponding monophenol are described.

2 The results of studies of the course of oxygen uptake, *o*-diphenol formation and disappearance of ascorbic acid during the oxidation by tyrosinase, in the presence of ascorbic acid, of tyrosine, phenol, *p*-cresol and 4,5-dimethylphenol (3,4-dimethylphenol) are reported.

3 The primary oxidation of monophenol to *o*-diphenol by tyrosinase in the presence of ascorbic acid was found to proceed at maximal rate at the moment of addition of enzyme to substrate. There was no induction period such as is observed in the absence of ascorbic acid. This leads to the conclusion that *o*-diphenol plays no specific part in facilitating the oxidation of monophenols by tyrosinase.

4 The behaviour of the reacting system containing initially monophenol, tyrosinase and ascorbic acid can be adequately explained in terms of (a) specific monophenolase activity of the enzyme preparation, (b) specific catecholase activity of the enzyme preparation, (c) progressive inactivation of the enzyme during the reaction, (d) competitive inhibition of catecholase activity by monophenols.

5 The bearing of the results on current views of the nature and action of tyrosinase is discussed. It is suggested that they support the view that tyrosinase is a single enzyme or enzyme complex having independent centres associated specifically with its monophenolase and with its catecholase functions.

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Polysaccharides Synthesized by Aerobic Mesophilic Spore-forming Bacteria

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In the classification proposed by Smith, Gordon & Clark (1946) the genus *Bacillus* has been divided into three groups depending principally on morphological differences in the size and shape of the spores and the shape of the sporangia. Their classification and nomenclature has been used throughout this paper.

The bacilli of group I are known to synthesize fructosans from sucrose, those obtained from *B subtilis*, *B megatherium* and *B pumilus* (*mesentericus*) have been intensively studied (Harrison, Tarr & Hibbert, 1930, Challinor, Haworth & Hirst, 1934, Lyne, Peat & Stacey, 1940, Evans & Hibbert, 1946) and all have been shown to be levans of the same general structure containing D-fructose as the sole constituent sugar.

The same attention has not been paid to the polysaccharides produced by the bacilli of group II. *B polymyxa* has been shown to produce a levan from sucrose, and an unidentified polysaccharide which is not a levan from other sugars (Hestrin, Avineri-Shapiro & Aschner, 1943). Kleczkowska, Norman & Snieszko (1940) isolated a new capsulated bacillus from cultivated soil which they named *B krzemienewski*. In some strains well defined capsules were present, while in others the mucilage was dispersed throughout the medium. Kleczkowski & Wierzchowski (1940) reported the separation of the capsular polysaccharide, and solely on the optical rotation of the total hydrolysate and the isolation of mannose phenylhydrazones, deduced that the polysaccharide was a mannan composed of L-mannose units. The sugar L-mannose has not been known to occur previously, and it has been commented (Evans & Hibbert, 1946) that such a result requires confirmation. *B krzemienewski* is now considered by Smith *et al.* (1946), pending the study and isolation of more strains, as a mucoid stage of growth of *B circulans*. It is classified as *B circulans* 760 in their collection. Group III bacilli do not appear to produce exocellular polysaccharides.

It was not our intention to make an intensive structural analysis of any of the polysaccharides produced by the bacilli, but rather, by studying the nature of the constituent sugars, to discover if any correlation exists between the groups of the above and the type of polysaccharide synthesized in presence of various sugars.

EXPERIMENTAL

Cultures We are grateful to Dr Ruth E. Gordon, the Curator of the American Type Culture Collection, for her kindness in sending us the following strains of polysaccharide-producing bacilli from their collection.

Group I			
	Strain no		Strain no
<i>B megatherium</i>	697	<i>B subtilis</i>	1248
<i>B pumilus</i>	355	<i>B cereus</i>	793
Group II			
<i>B polymyxa</i>	354	<i>B circulans</i>	294
<i>B brevis</i>	799	<i>B circulans</i>	295
<i>B alvei</i>	683	<i>B circulans</i>	
		<i>B macerans</i>	396
		intermediate	

A culture of *B circulans* strain no. 760 (*B krzemienewski*) was also sent. The culture, as received, was a mixture of the smooth and mucoid stages. These were separated on nutrient agar and designated for convenience *Km* (mucoid stage) and *Ks* (smooth stage). Both were tested for gum production on medium II of Cooper & Preston (1937). *Km* produced the typical colonies depicted by Kleczkowska *et al.* (1940), resembling glass beads in appearance, firm but resilient, and adhering strongly to the medium. *Ks* also produced gummy colonies on this medium, but the colonies were not firm, and the growth soon coalesced to form a gummy mass on the surface of the plate. The mucoid stage was unstable and slowly reverted to the smooth stage either on frequent subculture on Cooper & Preston's medium or after prolonged incubation or storage on this medium. It seems probable that *Km*, which is capsulated, is the same as strain A of Kleczkowska *et al.* (1940), while *Ks* corresponds to their strain B. These American cultures, Dr Gordon informs us, have been in storage for 6–10 years. In addition to the above, the following cultures of *B polymyxa* were available for study: *B polymyxa* NCTC, nos. 1380, 4745, 4744, and a strain of *B polymyxa* isolated in Prof. Kluyver's laboratory (obtained through the kindness of Dr Swaby of Rothamsted). Culture no. 4744 was that used by Hestrin *et al.* (1943).

Polysaccharide production For the preparation of sufficient polysaccharide for analysis of the constituent sugars, cultures of each organism were grown on the surface of agar in four 9 cm Petri dishes. The dishes, each containing 15–20 ml of Cooper & Preston's (1937) basic medium II with 2% of the various sugars, sterilized separately, were heavily inoculated and incubated at 25° for 2–7 days, depending on the organism. The surface growth was removed by means of

a sterile bent glass rod after the addition of a minimal quantity of sterile distilled water to facilitate harvesting. With *Km*, because of the toughness of the colonies and their adhesion to the agar surface, it was often necessary to leave the distilled water in contact with the surface growth for 24 hr in the incubator. This was usually sufficient to detach the colonies, which could then be removed without any danger of scraping up pieces of agar. Frequent tests subsequently showed that with reasonable care no contamination from the agar would be encountered. Polysaccharide synthesis by each organism was tested on sucrose, D-fructose, D-glucose, D-galactose, and L-arabinose. With each bacillus, polysaccharides containing identical sugar constituents were produced from the last four sugars, although in different yields. For brevity, description will thus be limited to the polysaccharides produced on sucrose and monosaccharides.

Partial purification of polysaccharides The growth, washed off with distilled water from four Petri dishes, was precipitated by dropping into 4 vol of ethanol containing 2% glacial acetic acid and stirring vigorously. After standing for 1 hr the precipitate was filtered on a sintered glass funnel and washed with ethanol of increasing strength, and then with ether. When dried in this manner the crude gums were generally in the form of fine white powders which could be redissolved in distilled water. Impurities could be spun down, and the polysaccharide reprecipitated with ethanol-acetic acid and dried as before. Where the mucilages were not readily dissolved in water, it was sometimes necessary to redissolve in 0.1N-NaOH. The products generally contained a considerable quantity of nitrogenous material derived from the bacteria. While the yield of this gum varied with the bacillus and the substrate sugar, in general c. 50-150 mg were obtained from the four Petri dishes.

Hydrolysis of the polysaccharides Two hydrolyses were carried out on each product: (a) c. 20 mg gum were hydrolyzed with 0.5 ml 0.1N-H₂SO₄ for 30 min in a sealed ampoule in a water bath at 100°, (b) c. 20 mg gum were hydrolyzed with 0.5 ml. N-H₂SO₄ for 4 hr as above. After the completion, the seals were broken and the hydrolysate neutralized to Congo red with solid BaCO₃. The Ba salts were then centrifuged down, still in the ampoule, and the supernatant liquid used for determination of sugars. The short hydrolysis in 0.1N H₂SO₄ serves to show the presence of furanose sugars such as D-fructose which would be almost totally destroyed in the stronger hydrolysis. When fructose was found to be present, the remainder of the gum was generally hydrolyzed with 0.5% oxalic acid for 1 hr on a water bath and the fructose-free residue, if present, was precipitated with ethanol, washed and dried as before. The residue could then be hydrolyzed and analyzed without interference from fructose and its decomposition products.

Identification of constituent sugars The sugars present were identified by the paper chromatographic technique (Partridge, 1946, 1948; Forsyth, 1948). From the *R_F* values of the constituent sugars using *n*-butanol, phenol, and *s*-collidine as solvents, by comparison with the position of standard sugars on the papers and by spraying with resorcinol and naphthoresorcinol (Forsyth, 1948), it was possible to identify all the sugars which were encountered. Uronic acids were detected both by the naphthoresorcinol test on the gum itself, and by spraying chromatograms of the hydrolysates with the reagent. In some cases quantitative estimations of sugars were made, after extraction from chromatograms, by the method of Flood, Hirst & Jones (1947).

RESULTS

Polysaccharides of Bacillus cereus 793, Bacillus subtilis 1248, Bacillus pumilus 355

When grown on sucrose these bacilli produced copious yields of gum, but no synthesis took place on a monosaccharide substrate. On hydrolysis with 0.1N-sulphuric acid or 0.5% oxalic acid, no appreciable residue of ethanol-insoluble material remained, and only one spot was obtained on paper chromatograms, which was readily identified as fructose from its *R_F* values (*n*-butanol 0.10, phenol 0.51, *s*-collidine 0.42) and its colour reaction on spraying with naphthoresorcinol. That D-fructose was the reducing sugar present was readily confirmed in each case by measuring the optical rotation and the reducing sugar value of a hydrolysate of the mother sample of the gum.

Polysaccharide of Bacillus megatherium 697

This bacillus produced polysaccharides on both the sucrose and monosaccharide media. The gum from the sucrose substrate, on hydrolysis with the 0.1N-sulphuric acid gave only D-fructose, identified as before. In the more concentrated N-sulphuric acid, however, the fructose was mainly destroyed giving only a faint spot, whereas another sugar was present which proved to be glucose. After hydrolysis in oxalic acid c. 40% of ethanol-insoluble residue still remained, which on further hydrolysis with N-sulphuric acid gave a hydrolysate free from fructose, but containing the other sugar as well as uronic material. This sugar was shown by spraying with resorcinol to be an aldose and was readily identified as glucose from its *R_F* values (*n*-butanol 0.07, phenol 0.39, *s*-collidine 0.39). This was confirmed by the preparation of glucosazone from the hydrolysate of the fructose-free residual polysaccharide. The gum from the monosaccharide media contained no fructose, giving only glucose and uronic material on hydrolysis. The presence of uronides was confirmed by the naphthoresorcinol test. It is probable that the gum produced on sucrose media is a mixture of levan and the glucose-uronic polysaccharide, which is synthesized alone on the monosaccharide media.

When grown on the liquid media of Tarr & Hibbert (1931) with the addition of 2% yeast extract and with sucrose as substrate sugar, only levan was produced, no glucose being detected, and the gum was completely hydrolyzed with oxalic acid. This phenomenon has been more closely investigated with *B. polymyxa*.

Polysaccharide of Bacillus polymyxa

All the strains of *B. polymyxa* investigated gave the same results. When grown on monosaccharide media a fructose-free polysaccharide was obtained,

which on hydrolysis with N-sulphuric acid, but not with 0.1 N-sulphuric acid, gave a hydrolysate shown by chromatography to contain two sugars. One of these was glucose and the other was identified as mannose from its R_F factors (*n*-butanol 0.10, phenol 0.45, *s*-collidine 0.46) and resorcinol spray, which showed it to be an aldose. This was confirmed by the preparation of mannose phenylhydrazone from a hydrolysate of the remainder of the gum. Uronides were also present.

When grown on sucrose medium a gum was obtained, which on hydrolysis with dilute acid gave fructose, identified as before. On hydrolysis with stronger acid a more complex sugar mixture was formed. With *n*-butanol two spots were present with R_F factors corresponding to glucose and fructose, but with phenol and *s*-collidine three spots were apparent. Apart from the glucose and fructose, mannose was also identified. Mannose and fructose have the same R_F factor in butanol. After removal of the fructose with oxalic acid, the residual polysaccharide gave the same sugars on hydrolysis as were produced from the polysaccharide formed on the monosaccharide substrate. As in the case of *B. megatherium*, a much larger proportion of levan was formed when *B. polymyxa* was grown on Tarr & Hibbert's liquid medium.

Dependence of levan production on culture media

As we have seen, both *B. megatherium* and *B. polymyxa* produce, from sucrose on Tarr & Hibbert's (1931) liquid medium, mainly levan, whereas on Cooper and Preston's (1937) solid media a considerable proportion of other polysaccharides, which are the sole products from a monosaccharide substrate, is produced. This difference may be due to one, or more than one, of the following factors: (1) aeration differences between solid and liquid culture, (2) differences in sucrose concentration (2% in Cooper & Preston's media and 10% in that of Tarr & Hibbert), (3) intrinsic differences in the basic media such as phosphate concentration.

Accordingly, *B. polymyxa* was grown under a variety of conditions, and levan estimated as reducing sugar after hydrolysis of the gums with oxalic acid. The residual ethanol-insoluble polysaccharide was also estimated by isolation and weighing. The results are shown in Table 1. The residual polysaccharide, even when only present in traces, gave glucose and mannose on hydrolysis. It is apparent from Table 1 that the aeration factor plays no part in levan production. Indeed a similar mixture of polysaccharides is produced when *B. polymyxa* is grown anaerobically. High concentrations of both sucrose and phosphate appear to be necessary for optimal levan production.

Table 1 *Levan production by Bacillus polymyxa*

Basic media	Type of culture	Sucrose (%)	PO ₄ (g/l)	Levan (% of total gum)	Residual gum as % of total gum
Cooper & Preston (1937)	Liquid	2	0.11	44	43
	Solid	2	0.11	39	39
	Solid	10	0.11	37	41
	Solid	10	0.53	69	13
Tarr & Hibbert (1931)	Liquid	10	0.53	86	7
	Solid	10	0.53	70	4
	Solid	2	0.53	37	40
	Solid	10	0.11	57	25

Polysaccharides of Bacillus brevis 799, Bacillus alvei 683

These strains produced polysaccharides containing the same sugars on both sucrose and monosaccharide substrates. No levan was formed. On hydrolysis with N-sulphuric acid, glucose and a uronide fraction were the only sugars identified.

Polysaccharides of Bacillus circulans 294, Bacillus circulans, Bacillus macerans intermediate 396

These bacilli, like the last two and all subsequently to be discussed, produced polysaccharides containing the same sugars on both sucrose and monosaccharide substrates and did not synthesize levan. Hydrolysis of the gum with N-sulphuric acid gave two sugars which were identified as before from their R_F factors and resorcinol spray as glucose and mannose. Uronides were also present.

Polysaccharide of Bacillus circulans 295

Hydrolysis of the gum produced by this bacillus with N-sulphuric acid gave a mixture of three sugars as well as uronic residues. Two of the sugars were readily identified as before as glucose and mannose. The third sugar was shown to be a pentose by resorcinol spray and was identified as xylose from its R_F factors (*n*-butanol 0.13, phenol 0.44, *s*-collidine 0.50). Comparison was made on chromatograms with the other three pentoses, arabinose, ribose, lyxose, in order to confirm that the sugar was xylose. The presence of xylose was further confirmed by the preparation of the dimethyl acetal of dibenzylidene xylose (Bredy & Jones, 1945; Wise & Ratchiff, 1947) from the sugar syrup prepared from a hydrolysate of the remainder of the gum.

Polysaccharides of Bacillus circulans 760 (Bacillus krzemieniewski)

These have been most carefully studied since it has been claimed (Kleczkowski & Wierzchowski, 1940) that a mannan composed of L-mannose units is the main polysaccharide synthesized.

Ks (smooth form) The strain was grown on a range of sugars and the crude gum isolated as for the other bacilli previously discussed. A rough estimate of the production of polysaccharide by the strain on different sugars may be gained from a comparison of the yields of this partly purified product shown in Table 2. The gums contained no levan, and on hydrolysis gave glucose and mannose, identified chromatographically as before.

Table 2 *Yields of gum by Bacillus circulans 760 (smooth form) on different sugars*

(Growth at 25° for 7 days)

	mg /plate		mg /plate
Sucrose	108	D Galactose	24
Mannitol	62	Lactose	24
D Glucose	58	L Arabinose	14
D Fructose	46	L Rhamnose	4

Km (mucoid form) Similar analysis was made on the polysaccharide synthesized by the well-capsulated form of the organism and the same constituent sugars were found to be present. To exclude the possibility that this polysaccharide, which is of a more complex nature than a simple mannan, is synthesized because of different culture conditions, both *Ks* and *Km* were grown on the media used and the conditions prescribed by Kleczkowski & Wierzchowski (1940). Again both glucose and mannose were detected in the hydrolysates.

For further investigation larger amounts of gum were prepared from *Ks*, and further purified in a similar manner to that described by Kleczkowski & Wierzchowski (1940). 5 g of the gum were heated under reflux with 300 ml *N* KOH for 3 hr on a water bath at 100°, and the hot solution filtered through glass wool and then centrifuged. On cooling a very viscous gel formed. The alkaline solution was poured into 4 vol. of ethanol containing acetic acid, washed with ethanol, then ether, and dried. When purified by electrodialysis, as described by Kleczkowski & Wierzchowski, a low yield of purified product was obtained. Nevertheless, this method was used with a portion of gum to obtain sufficient material for sugar analysis. On hydrolysis of the electrodialyzed polysaccharide, glucose and mannose were formed as before. The bulk of the material was further purified in good yield by forming the copper complex by the addition of CuSO_4 to an alkaline solution of the gum. The complex was washed with hot water, and then decomposed with ethanol containing HCl and washed free from Cu^{++} and Cl^- ions with ethanol. After drying with ether, the regenerated gum was redissolved in a small volume of 0.1 *N* NaOH and precipitated by a large excess of glacial acetic acid. After drying with ethanol and ether the product (1.45 g) was a fine white powder, difficultly soluble in water to give an opalescent, very viscous solution. It was readily soluble in dilute alkali and was almost free from ash and N. After electrodialysis it gave an equivalent weight of 598 on titration with 0.1 *N*-NaOH using phenolphthalein as indicator. The equivalent weight was not significantly altered on fractional precipitation of the polysaccharide with ethanol. Determination of uronic anhydride

by CO_2 evolution on refluxing with 12% HCl gave a value of 28.9% (calculated from equivalent weight, 29.8%). This uronic acid would give rise to c. 6.3% furfural (found, 6.1%) which confirms the absence of pentoses. Glucose, mannose, and a uronic acid were again shown to be present by paper chromatography.

The sugars on the paper chromatograms were determined by the method of Flood *et al.* (1947). Glucose/mannose ratio (Exp. 1) 1.53, (Exp. 2) 1.45. Using a known weight of xylose as an internal standard, the actual percentages of glucosan and mannan were determined (Table 3). Mannose was also estimated as the phenylhydrazone by the method of Jones, Hirst & Woods (1947).

Table 3 *Composition of purified gum from mucoid form of Bacillus circulans 760*

	Glucosan (%)	Mannan (%)	Uronic anhydride (%)	Equv. wt
Theoretical	41.8	27.9	30.3	581
Found	40.5	25.6	28.9	598
		25.8*		

* As phenylhydrazone

Hydrolyses with 0.2% oxalic acid for 4 hr or with 0.1 *N* H_2SO_4 for 30 min at 90° were not effective in splitting off reducing sugars, suggesting the absence of furanose sugars. 1.0 g polysaccharide was refluxed with 50 ml *N*- H_2SO_4 for 3 hr, and the change in rotation determined (1 hr, $[\alpha]_D^{20} +29.5^\circ$, 2 hr, $+33.3^\circ$, 3 hr, $+33.3^\circ$). While this rotation is of the right order for a mixture of D uronic acids, D glucose and (?) mannose, such as are produced, it is in direct contrast to that of Kleczkowski & Wierzchowski, who obtained a negative rotation. The hydrolysate was neutralized with BaCO_3 , centrifuged and concentrated. On addition of methanol no barium aldobionate fraction separated. The solution was concentrated to a syrup and taken up in 10 ml water and mannose phenylhydrazone prepared, m.p. 192°. The hydrazone was then decomposed by boiling in water with a slight excess of benzaldehyde, and, after filtration and removal of the excess benzaldehyde with ether, the regenerated mannose was shown by optical rotation, $[\alpha]_D^{20} = +14^\circ$, to be D mannose. That mannose was the sugar regenerated was confirmed by paper chromatography.

DISCUSSION

The results obtained are tabulated in Table 4. It is apparent that levan formation is confined to group I bacilli, with the exception of *Bacillus polymyxa*, and the synthesis of other polysaccharides to group II bacilli, with the exception of *B. megatherium*. *B. polymyxa* and *B. megatherium* might be considered as intermediate since they produce varying proportions of both levan and other polysaccharides from sucrose depending on the culture conditions. *B. circulans* strains produce polysaccharides containing glucose, mannose and a uronic acid. The nature of the polysaccharide is independent of the substrate sugar. It is, of course, by no means proved that these complex polysaccharides are not merely

mixtures of simple dextrans, mannans and poly-uronic acids, but attempts to fractionate them by fractional ethanol precipitation have proved unsuccessful We have not been able to confirm Kleczkowski & Wierzchowski's (1940) claim that *B krzemienniewski* produces a mannan composed of L-mannose units The strains available synthesized complex polymers of glucose, mannose and uronic acid in the approximate ratio 3 2 2 The mannose was D-mannose and not the unnatural L-form *B circulans* 295 gum is unique in that it contains the pentose, xylose

Table 4 Constituent sugars of the mucilaginous polysaccharides synthesized by the genus *Bacillus*

Bacilli		Levan formation on sucrose	Other polysac- charides synthesized on sucrose and monosaccharides
Group	Species		
I	<i>cereus</i>	793	—
	<i>pumilus</i>	355	
	<i>subtilis</i>	1248	
	<i>megatherium</i>	697	
II	<i>polymyxa</i>	354	Glucose uronic
	<i>circulans</i>	760	
	<i>circulans</i>	294	
	<i>circulans</i>	396	
	<i>macerans</i>		Glucose-mannose- uronic
	<i>brevis</i>	799	
	<i>alvei</i>	683	
	<i>circulans</i>	295	

It may be of some interest that when a crude ethanol precipitate of a culture of these bacilli is hydrolyzed, ribose, presumably from the nucleic

acids, is readily identified among the polysaccharide sugars

SUMMARY

1 The constituent sugars of polysaccharides synthesized by the genus *Bacillus* from sucrose and monosaccharide sugars have been identified Strains of *B cereus*, *B pumilus*, *B subtilis*, *B megatherium*, *B polymyxa*, *B circulans*, *B circulans-macerans*, *B brevis*, and *B alvei* have been studied

2 There appears to be a relation between type of polysaccharide synthesized and the group classification as proposed by Smith *et al* (1946) Levan synthesis from sucrose is confined to group I bacilli and *B polymyxa* On the other hand, the synthesis of other polysaccharides from sucrose and monosaccharides is confined to group II bacilli and *B megatherium*

3 *B polymyxa* and *B megatherium* are intermediate in that they synthesize both levan and other polysaccharides from sucrose depending on the culture medium

4 Group II bacilli synthesize from a wide variety of sugars polysaccharides containing (a) glucose and a uronic acid, (b) glucose, mannose, and a uronic acid, (c) glucose, mannose, xylose, and a uronic acid The type of polysaccharide is characteristic of the bacillus and not of the substrate sugar

5 We have not been able to confirm Kleczkowski & Wierzchowski's (1940) claim that *B krzemienniewski* produces a mannan composed of L-mannose units The strains available synthesized a complex polymer of glucose (3 parts), mannose (2 parts) and uronic acid (2 parts) The mannose was the 'natural' D-mannose

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The Pathway of the Adaptive Fermentation of Galactose by Yeast

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Certain yeasts can ferment galactose by the formation of an adaptive 'galactozymase' when placed in aqueous solutions of galactose and in the apparent absence of cell division (Stephenson & Yudkin, 1936, Spiegelman, 1945). Though much work has been done on the mechanism of this adaptation (Spiegelman, 1947) little knowledge has so far been obtained concerning the actual enzymic changes involved or of the intermediates formed.

Kosterlitz (1943*b*), who has reviewed the information available, showed that a maceration juice prepared from galactose-adapted yeast would ferment both α glucose 1-phosphate and α galactose-1-phosphate in a similar manner, he suggested that α -galactose 1-phosphate played the same role in galactose fermentation as did α -glucose-1-phosphate and glucose 6 phosphate in glucose fermentation. Working with dialyzed yeast extracts, Kosterlitz showed that 27% of the α -galactose-1-phosphate added was converted into a difficultly hydrolyzable ester. In the same time, about 90% of α -glucose-1-phosphate was converted to glucose-6-phosphate. To explain these results, he suggested that at least two new enzymes were involved in galactose fermentation: one converting galactose to α galactose 1-phosphate, and a second converting this ester into α glucose-1 phosphate which is then transformed into the Robison ester.

The work presented in this paper describes the preparation of an extract from galactose adapted yeast and the formation of an easily hydrolyzable phosphoric ester from galactose and adenosinetriphosphate in the presence of this extract. This ester has been identified as α -galactose-1-phosphate. Some evidence is also presented in favour of the view that galactose 1-phosphate is further transformed into glucose-6 phosphate.

EXPERIMENTAL

Organisms, growth media and preparation of extracts The work was carried out with a strain of Dutch top yeast (Cat no 174) obtained from the Carlsberg Laboratories. The yeast was grown in Roux bottles, each of which contained 150 ml of the following medium: 0.1% $\text{NH}_4\text{H}_2\text{PO}_4$, 0.1% $(\text{NH}_4)_2\text{HPO}_4$, 0.2% KH_2PO_4 , 0.2% Difco Yeast Extract, 2% galactose, 1% of a trace element solution. The trace element solution contained, per litre: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20.0 g,

NaCl , 1.0 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g, $\text{MnSO}_4 \cdot 3\text{H}_2\text{O}$, 0.5 g, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.05 g, 0.1N H_2SO_4 , 10 ml.

The medium was sterilized and inoculated from a 24 hr culture of the yeast in the above medium at 25°. After 36 hr at 25° the yeast was harvested, washed on the centrifuge with 0.05M NaH_2PO_4 , and made into a thick suspension in a solution containing 0.1% galactose and 0.01% neutralized Na_2S . The suspension was then shaken with glass beads for 15 min on a Mickle disintegrator (Mickle, 1948). It was found necessary to carry out the disintegration under anaerobic conditions in order to obtain fully active extracts: such conditions were obtained by the addition of 0.01% neutralized Na_2S to the suspension, and by filling the gas space in the vessels of the disintegrator with H_2 . The glass beads were filtered off on a coarse sintered glass filter funnel, and the filtrate centrifuged at 3000 r.p.m. for 15 min. The cell free supernatant fluid was used for the experiments.

Reagents and methods The adenosinetriphosphate (ATP) used was made from a commercial preparation of the barium salt supplied by Boots Ltd. This was purified by dissolving it in 0.2N HNO_3 until the solution was just acid to Congo red, and then by following the final stages of the preparation described by Needham (1942). Galactose 6 phosphate was prepared according to the method of Levene & Raymond (1931), and α glucose 1 phosphate by the method of Hanes (1940). Glucose and galactose solutions were prepared from the pure bacteriological sugars supplied by Kerfoot's Ltd.

Acid production The phosphorylation of hexose by ATP in the 1 or 6 position results in an increase in acidity of the reaction mixture. Phosphorylation can therefore be followed manometrically by measuring the CO_2 evolved when the reaction is carried out in a bicarbonate CO_2 buffer system (Colowick & Kalckar, 1943). Warburg manometers were used for this purpose.

Reducing sugar was estimated by the method of Semogyi (1945). Samples were first deproteinized by means of ZnSO_4 and $\text{Ba}(\text{OH})_2$, which also removes any phosphorylated hexose esters. Fructose was determined by the method of Roe (1934), and pentose by the method of Maybaum (1939). The distribution of P in the various fractions was determined by estimation of orthophosphate by the Fiske & Subbarow method (1925), as follows: direct estimation, $P_{0\text{min}}$ = ortho P, after wet ashing = total P, after hydrolysis in N HCl at 100° for 7 min, $P_{7\text{min}}$, by calculation, $P_{7\text{min}} - P_{0\text{min}}$ = acid labile P, total P - $P_{7\text{min}}$ = acid stable P.

Separation of the Ba salts of the phosphoric esters formed during the reaction was carried out according to the procedure of Umbreit, Burris & Stauffer (1945).

Differential analysis of 'glucose' and 'galactose' by fermentation was carried out by a modification of the method of Harding & Grant (1931-2). Dutch top yeast was used in

place of baker's yeast, and the disappearance of sugar was followed manometrically by the anaerobic CO_2 evolution. When this was complete, the residual sugar was measured by the method of Somogyi (1945).

RESULTS

Preliminary experiments with the crude extract

Neither glucose nor galactose was fermented by the crude extract, even after the addition of boiled yeast extract, hexosediphosphate, acetaldehyde and ATP, singly or in combination, hexosediphosphate itself was not fermented. The ability of the extract to phosphorylate glucose or galactose in the presence of ATP was tested by following the increase in acidity in bicarbonate buffer, and the results were checked at the end of the reaction by measurement of the free sugar disappearing. The results are shown in Table 1.

Table 1 *Effect of adenosinetriphosphate on the increase in acidity (CO_2 output) and sugar disappearance on addition of glucose and galactose to extracts prepared from normal and galactose-adapted Dutch top yeast*

(The manometers contained—Main chamber extract, 0.6 ml, 0.5M-NaF, 0.2 ml, 0.12M NaHCO_3 , 0.5 ml, 0.1M- MgCl_2 , 0.2 ml. Side bulb 0.12M NaHCO_3 , 0.3 ml, and, where added, glucose or galactose (20 mg/ml) 0.2 ml., ATP (2.5 mg $\text{P}_{7\text{min}}/\text{ml}$), 0.4 ml. Gas phase $\text{N}_2 + 5\% \text{CO}_2$, temp 25° . Incubation time 30 min, after which the free sugar was measured on a 1 ml sample.)

	Crude extract		Purified extract	
	Normal	Galactose adapted	Normal	Galactose adapted
CO_2 output (μmol)				
Control	0.18	0.18	0.09	0.09
Control + ATP	7.06	7.59	2.05	1.61
Glucose	0.80	0.98	0.09	0.18
Glucose + ATP	10.52	11.68	8.72	8.39
Galactose	0.01	0.00	0.00	0.09
Galactose + ATP	6.97	12.91	2.18	8.35
Sugar disappearance (μmol)				
Glucose	0.00	0.56	0.00	0.00
Glucose + ATP	8.78	9.12	12.92	14.21
Galactose	0.00	0.00	0.00	0.00
Galactose + ATP	0.22	10.00	0.00	13.98

There is no significant CO_2 output or sugar disappearance in the absence of ATP, whereas in its presence there is a considerable CO_2 production in the control, which is significantly increased in presence of glucose or galactose in the case of the extract prepared from galactose-adapted yeast. In the case of the extract from yeast grown in glucose there is an increased CO_2 production over the control only when glucose is added. The increased CO_2 production after the addition of glucose or galactose can be correlated with the disappearance of sugar in

both instances. The large CO_2 output in the control was undesirable, and to minimize this it was necessary to effect some purification of the enzymes concerned.

Purification of the extract Attempts to remove interfering sugars by dialysis resulted in inactivation of the enzyme system attacking galactose. Even dialysis overnight at 0° against an aqueous solution containing 1% galactose and 0.1% neutralized Na_2S resulted in a complete loss of activity, which could not be restored by the addition of cysteine or of a boiled extract from either normal or galactose-adapted yeast, singly or together. Hexokinase was not inactivated by this dialysis.

The extract was, therefore, fractionated by precipitation, and the following procedure was ultimately adopted. The extract was cooled to 0° and the pH brought to 4.4 by the addition of M acetate buffer. The precipitate was separated on the centrifuge and discarded. The supernatant fluid was adjusted to pH 7.5 with 0.1N NaOH and solid $(\text{NH}_4)_2\text{SO}_4$ added to bring the final concentration to 75% saturation. After centrifuging, the precipitate was dissolved in a small volume of distilled water and used for the following experiments.

The CO_2 output in absence of sugar was largely eliminated by the purification, as can be seen from Table 1. The increased CO_2 production, after the addition of glucose or galactose, can again be correlated with the disappearance of sugar.

Activation of the enzyme phosphorylating galactose During the purification process the enzyme lost some activity, which was largely restored by the addition of Mg^{++} , a further increase in activity resulting on the addition of cysteine (Fig. 1). Thus, this system resembles all known phosphokinases in requiring Mg^{++} , and in being activated by thiol compounds.

The presence of phosphoglucomutase and phosphohexose-isomerase in the extract The presence of these enzymes in the extract was tested as follows. Manometers were set up containing—Main chamber glucose 1-phosphate (4.0 mg $\text{P}_{7\text{min}}/\text{ml}$), 0.7 ml, 0.5M NaF, 0.3 ml, 0.12M- NaHCO_3 , 0.4 ml, 0.1M- MgCl_2 , 0.1 ml, 0.25M cysteine, 0.1 ml. Side bulb 0.1M- NaHCO_3 , 0.25 ml, extract, 0.75 ml (boiled extract, 0.75 ml in control).

The manometers were filled with a CO_2/N_2 mixture containing 5% CO_2 , and the contents of the side bulbs tipped into the main cup after equilibration. After shaking for 90 min, 2 ml of the contents were taken for analysis as follows. 1 ml of 10% trichloroacetic acid was added to each sample, and the precipitate centrifuged and washed with water. The supernatant fluid and washings were combined, brought to pH 7.5 with 10% NaOH, and diluted to 10 ml. Determinations were then made of total P, acid labile P (corresponding to glucose 1-phosphate P), and of the fructose content from which the P present as fructose 6-phosphate was calculated. Aldose 6-phosphate P was then calculated as (acid stable P—fructose 6-phosphate P).

In the control experiment with the boiled extract, 92% of the glucose-1-phosphate was unchanged at the end of the experiment, with the active extract an equilibrium mixture was obtained consisting of 15%

glucose-1-phosphate, 26 % fructose-6-phosphate and 59 % aldose-6-phosphate. Thus phosphoglucomutase and phosphohexoseisomerase were present in the extract.

The distribution of phosphoric esters in the products of the reaction between adenosinetriphosphate and glucose or galactose

Manometers were set up as described in Fig. 2, and the following quantities were measured during the course of the reaction: (1) increase in acidity, by the CO_2 evolution in bicarbonate buffer, (2) sugar disappearance, (3) change in orthophosphate, and (4) change in acid-labile phosphate.

It can be seen from Fig. 2 that the increase in acidity roughly paralleled the disappearance of either glucose or galactose. With glucose (Fig. 2A) there was a decrease of 0.475 mg in the amount of acid-labile P, corresponding to an esterification of 2.81 mg glucose, while actually 3.2 mg glucose disappeared. Thus 87.5 % of the glucose phosphorylated can be accounted for as acid-stable phosphoric esters, presumably an equilibrium mixture of aldose 6-phosphate and fructose-6-phosphate. In the case of galactose, however, there was no significant decrease in the amount of acid-labile P during a period in which 2.1 mg of galactose disappeared. This suggests that an acid-labile ester such as galactose-1-phosphate was formed.

The disappearance of acid-labile P in the presence of glucose, less than that of the control, corresponds to more than one of the acid-labile phosphate groups of ATP. This is supported by the figures for the disappearance of glucose. Since yeast hexokinase is said to be specific to ATP, and to have no reaction with ADP (Colowick & Kalckar, 1943), this result

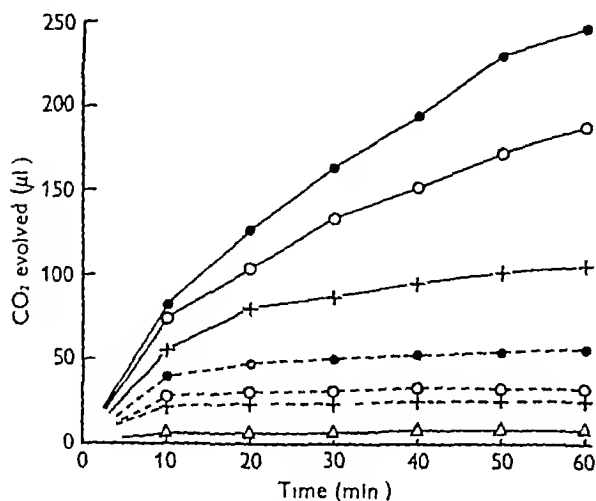


Fig. 1 Activation of the galactose phosphorylating system in an extract prepared from galactose adapted Dutch top yeast. The manometers contained—Main chamber: extract, 0.6 ml, 0.5 M-NaF, 0.2 ml, 0.12 M NaHCO_3 , 0.5 ml, and, where added, 0.1 M- MgCl_2 , 0.1 ml, 0.25 M cysteine, 0.1 ml. Contents made up to 1.5 ml with distilled water. Side bulb: 0.1 M- NaHCO_3 , 0.3 ml, and, where added, ATP (3.0 mg P_i min.), 0.4 ml, galactose (20 mg/ml), 0.2 ml. Contents made up to 0.9 ml with distilled water. Gas phase: 95 % N_2 + 5 % CO_2 , temp 25°. —, mixtures containing galactose; ---, mixtures without galactose; ●, ATP, Mg^{++} and cysteine; ○, ATP and Mg^{++} ; +, ATP only; Δ, Mg^{++} and cysteine.

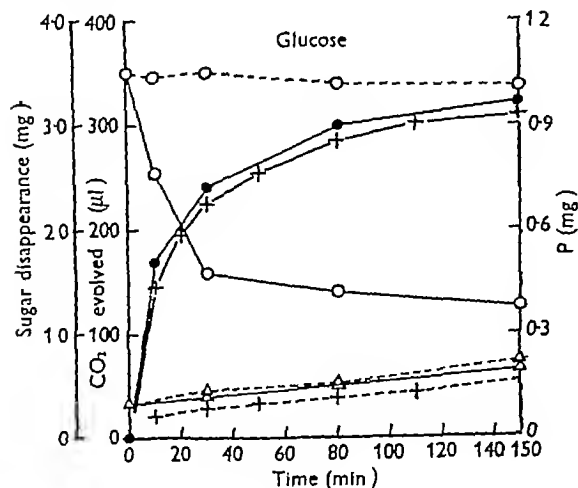


Fig. 2A

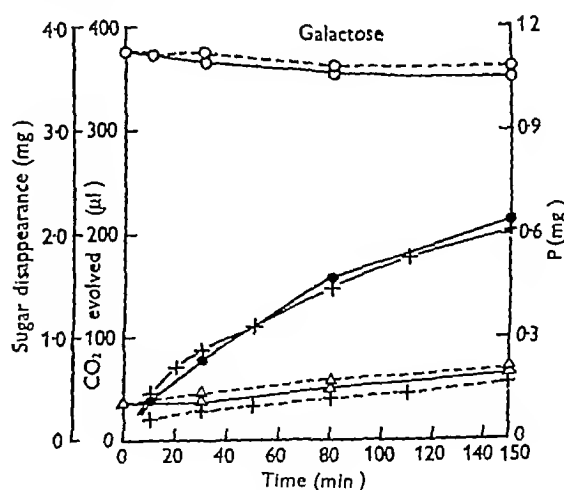
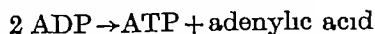


Fig. 2B

Fig. 2 Increase in acidity (CO_2 output), sugar disappearance and phosphate distribution during the breakdown of glucose or galactose by an extract prepared from Dutch top yeast grown on galactose. The manometers contained—Main chamber: extract, 0.6 ml, 0.5 M-NaF, 0.3 ml, 0.12 M NaHCO_3 , 0.7 ml, 0.1 M MgCl_2 , 0.1 ml, 0.25 M cysteine, 0.1 ml, glucose (20 mg/ml), 0.3 ml in A, galactose (20 mg/ml), 0.3 ml in B. Contents made up to 2.1 ml with distilled water. Side bulb: 0.12 M NaHCO_3 , 0.25 ml, ATP (2.5 mg P_i min./ml), 0.5 ml. Gas phase: 95 % N_2 + 5 % CO_2 , temp 25°. ---, control; —, glucose or galactose; ○—○, phosphate hydrolyzable in 7 min by HCl at 100°; ●—●, free sugar disappearance; +—+, increase in acidity; Δ—Δ, orthophosphate.

suggests the presence in the extract of a myokinase-like enzyme catalyzing the reaction



To confirm the production of an acid-labile phosphoric ester from galactose, an experiment was performed in which the distribution of P among the various phosphoric esters was determined by separation into soluble and insoluble barium salts, and estimation of inorganic P, acid-labile P, and total P in each fraction

The results are shown in Table 2 Exp A. When glucose is the substrate, the ATP-P which disappears (decrease in acid-labile P of insoluble barium salts), is largely accounted for in the soluble barium salts as an acid-stable ester (probably a

grown on glucose will not phosphorylate galactose. Berger, Sleim, Colowick & Cori (1945-6) have shown that purified yeast hexokinase will not attack galactose

Isolation of α galactose-1-phosphate

The reaction mixture contained 40 ml. ATP solution (approx 6 mg $P_{7 \text{ min.}}/\text{ml}$), 1 g galactose, 2 ml m-cysteine , 5 ml 0.1M- MgCl_2 , 20 ml 0.5M- NaF , 40 ml 0.12M- NaHCO_3 , 40 ml extract, and water to a total volume of 150 ml. Portions (50 ml.) were placed in each of three Krebs (1933) vessels which were filled with a N_2/CO_2 mixture containing 5% CO_2 and shaken in a bath at 25° . The reaction was allowed to proceed until no more galactose disappeared. Trichloroacetic acid (20 ml of 40%) was then added to the combined contents of the vessels, and the precipitate centrifuged and

Table 2 *Distribution of phosphoric esters formed by the action of an extract prepared from galactose-adapted Dutch top yeast on a mixture of galactose or glucose and adenosinetriphosphate*

(Exp A, normal extract, initial acid labile P = 3.62 mg. Exp B, extract prepared at 0° , initial acid labile P = 3.21 mg. The manometers contained—Main chamber 0.5M- NaF , 0.3 ml, 0.1M- MgCl_2 , 0.3 ml, 0.25M cysteine, 0.3 ml, 0.12M- NaHCO_3 , 1.4 ml, ATP (3-4 mg $P_{7 \text{ min.}}/\text{ml}$), 1.0 ml, and where added, glucose or galactose (20 mg/ml), 0.8 ml. Contents made up to 4.1 ml with distilled water. Side bulb extract, 0.5 ml., 0.5M- NaF , 0.1 ml, 0.12M NaHCO_3 , 0.3 ml. Gas phase 95% N_2 + 5% CO_2 , temp 25° . CO_2 output followed until complete (150 min in A, 280 min. in B). Contents of manometers then separated into soluble and insoluble barium salts and analyzed.)

	Soluble Ba salts					Insoluble Ba salts				Total P	
	Inorganic P (mg)	Acid labile P (mg)	Acid stable P (mg)	Increase in acid labile P* (mg)	Increase in acid stable P* (mg)	Inorganic P (mg)	Acid-labile P (mg)	Acid stable P (mg)	Decrease in acid labile P* (mg)	Initial (mg)	Recovered (mg)
Exp A											
Control	0.00	0.24	0.48	—	—	0.74	2.86	1.92	—	6.81	6.24
Glucose	0.04	0.42	1.78	0.18	1.30	0.74	1.38	1.68	1.48	6.81	6.04
Galactose	0.02	1.28	0.64	1.04	0.16	0.76	1.80	1.96	1.06	6.81	6.26
Exp B											
Control	0.00	0.12	0.19	—	—	0.31	2.50	1.56	—	4.82	4.37
Glucose	0.00	0.18	2.29	0.06	2.10	0.28	0.67	1.26	1.83	4.82	4.34
Galactose	0.00	1.13	1.00	1.01	0.81	0.36	0.96	1.16	1.54	4.82	4.25

* Compared with control.

mixture of aldose-6-phosphate and fructose-6-phosphate), and only a small amount of an acid-labile ester is found in this fraction (probably glucose-1-phosphate). When galactose is the substrate, the situation is reversed, and the major part of the ATP-P disappearing is recovered in the soluble fraction as an acid-labile ester. The proportions of acid-labile to acid-stable P in the soluble barium salts are 0.14/0.86 and 0.87/0.13 for glucose and galactose respectively.

The experiment described in Table 2, Exp A, was repeated using an extract prepared from non-adapted Dutch top yeast. In this case, 96% of the galactose remained unchanged after 150 min and the CO_2 evolution was 165 μl compared with 161 μl in the control. In the same time, 74% of the glucose disappeared and the corresponding CO_2 evolution was 871 μl . Thus it appears that Dutch top yeast

washed with 5% trichloroacetic acid. The supernatant and washings were brought to pH 8.5 with 10% NaOH , and barium acetate (50 ml of 25%) added. The precipitate was centrifuged and the supernatant poured off and kept. The precipitate was then dissolved in 25 ml 0.1N HCl , the pH brought to 8.5 with 10% NaOH and the precipitate centrifuged and discarded. The double extraction was necessary since the first precipitate contains some 'barium soluble' material in the form of double salts (Cori & Cori, 1932). Two vol ethanol were added to the combined supernatants, and the mixture left at 0° for 2 hr. The precipitate was then centrifuged, washed in turn with 75% ethanol, 96% ethanol and ether and dried *in vacuo* over CaCl_2 . From 0.57 g galactose metabolized, 3.18 g of crude soluble Ba salt were obtained, containing 124.1 mg P of which 73.4 mg was acid-labile.

The crude Ba salts contained an impurity of carbohydrate nature, which was removed by taking advantage of the solubility of the K salts of hexosemonophosphates at pH 6.0 in 60% ethanol, under these conditions, polysaccharides are

precipitated. The salt was taken up in 200 ml water, the insoluble material being discarded, the Ba^{++} removed from the supernatant by addition of a slight excess of saturated K_2SO_4 , and the pH adjusted to 6.0 with 10% KOH, 3 vol ethanol were then added. The mixture was left at 0° overnight, and the precipitate discarded. The supernatant was concentrated *in vacuo* to 40 ml, and the precipitate which formed again discarded. The supernatant was brought to pH 8.4 with 10% NaOH, and 5 ml 25% barium acetate and 2 vol ethanol were added. After leaving at 0° overnight, the precipitate was washed and dried in the usual manner, wt 1.281 g, P, 5.1%, of which 88.6% was acid labile. The recovery of acid labile P from the crude Ba salt was 79.2%. A sample of the material, after treatment with $\text{Ba}(\text{OH})_2$ and ZnSO_4 , was free from Cu reducing substance. Another sample was hydrolyzed for 7 min in N HCl, and the sugars so produced were subjected to a differential fermentation. Ba salt (50 mg, containing 2.27 mg acid labile P) yielded 12.9 mg of reducing sugar (equivalent to 2.25 mg P), of which only 8% was fermented by glucose grown yeast and a further 92% by galactose grown yeast. It is assumed, therefore, that 8% of the acid labile P was present as a glucose ester, and 92% as a galactose ester. The salt contained negligible amounts of pentose and fructose, the acid stable P probably being present largely as aldose 6-phosphate.

Any reducing phosphates were removed by alkaline hydrolysis as follows. The salt (1.136 g) was dissolved in 100 ml water and Ba^{++} removed with 5 ml N- H_2SO_4 . The precipitate was centrifuged and washed with 5 ml water. To the supernatant and washings were added 27 ml N-NaOH and hydrolysis was allowed to proceed at 100° for 30 min. After cooling, the solution was brought to pH 8.4 with 2N HCl, 25 ml 25% barium acetate added, and the precipitate collected in the usual manner. Further purification was effected by repeated solution in water and precipitation with ethanol. The final product was identified as barium α -galactose 1-phosphate as follows.

Identification of the product as barium α -galactose 1-phosphate. The salt was dried over P_2O_5 *in vacuo* to a constant weight of 474 mg, P, 7.49%, acid labile P, 7.47% (Calc for $\text{C}_6\text{H}_{11}\text{O}_6\text{PBA}$, P, 7.85%). The recovery of acid labile P from the crude barium salt was 48.1%. The product contained less than 0.1% N, no detectable reducing power as measured by the method of Somogyi (1945), and negligible amounts of pentose and ketose. Further evidence on its identity was obtained as follows.

(1) *Sugar liberated on hydrolysis.* Hydrolysis in N-HCl at 100° for 7 min resulted in the simultaneous liberation of reducing sugar and inorganic P in equimolecular amounts. On hydrolysis and differential fermentation of the sugars liberated, 25 mg Ba salt yielded 0.6 mg 'glucose' (2.4%), 10.2 mg 'galactose' (40.8%), and 1.76 mg inorganic P, giving a molecular ratio galactose:acid labile P of 0.99:1.00 (Barium galactose 1-phosphate requires galactose, 45.5%).

(2) *Specific rotation.* The Ba salt had $[\alpha]_{\text{D}}^{20} = +110^\circ$. This agrees well with the value found by Kosterlitz (1943a) of 109.5° for the Ba salt of synthetic α -galactose 1-phosphate, and with that of 113° for the Ba salt of the natural ester calculated by the same author from the rotation of an impure preparation. Reithel (1945) found the specific rotation of the barium salt of β -galactose 1-phosphate to be $[\alpha]_{\text{D}}^{20} = +34^\circ$.

(3) *Hydrolysis constant.* Hydrolysis was carried out in 0.25N-HCl at 25° using the K salt of the ester, the galactose

liberated being measured by the Somogyi (1945) method and the inorganic P by the Fiske & Subbarow (1925) method.

The hydrolysis constant in 0.25N-HCl at 25° was 0.86×10^{-3} (Table 3). The corresponding value for the K salt of synthetic α -galactose 1-phosphate was found by Kosterlitz (1939) to be 0.89×10^{-3} , and the same author (Kosterlitz, 1943a) calculated the hydrolysis constant for the K salt of the natural ester from the hydrolysis rate of an impure preparation to be 0.91×10^{-3} .

Table 3 *Acid hydrolysis of the acid labile galactose ester*

(Hydrolysis at 25° in 0.25N HCl. Initial concentration of acid-labile P = 2.93 mM. Initial concentration of galactose = 2.88 mM. The hydrolysis constant k was calculated

from the formula $k = \frac{1}{t_2 - t_1} \log_{10} \frac{a - x_1}{a - x_2}$)

Time (min)	Galactose liberated (% of total galactose)	$k \times 10^3$	Inorganic P liberated (% of total acid labile P)	$k \times 10^3$
100	18.2	0.87	18.3	0.88
200	32.7	0.85	32.9	0.86

On the basis of this evidence it is concluded that the easily hydrolyzable ester formed from galactose and ATP is α -galactose-1-phosphate.

Possible formation of galactose-6-phosphate as an intermediate

To test the possibility that the primary phosphorylated product in galactose fermentation is galactose-6-phosphate, which is immediately transformed to galactose-1-phosphate, the effect of adding synthetic galactose 6-phosphate to the enzyme system phosphorylating galactose was studied. The results are shown in Table 4. In the presence of galactose 6-phosphate, the distribution of phosphoric esters did not differ significantly from that of the control, and there was no diminution in the amount of acid stable P during the course of the reaction. These facts, together with the absence of increased acid formation (CO_2 evolution) over the control, indicate that galactose 6-phosphate is not attacked in a system which is able to form an easily hydrolyzable phosphoric ester from galactose. Galactose 6-phosphate is, therefore, not an intermediate in the formation of galactose-1-phosphate in this system.

The action of the enzyme system on D-tagatose, the ketose corresponding to D-galactose, was also investigated. Tagatose was not fermented by intact cells of galactose adapted yeast, but this does not necessarily exclude it from playing a part in galactose fermentation analogous to that of fructose in glucose fermentation. It can be seen, however, from Table 4, that tagatose was not phosphorylated by an extract of galactose-adapted cells. The figures for the P distribution in the presence of tagatose were almost identical with those in the control.

Table 4 *Distribution of phosphoric esters formed by the action of an extract prepared from galactose-adapted Dutch top yeast on a mixture of galactose, galactose-6-phosphate or tagatose, and adenosinetriphosphate*

(The manometers contained—Main chamber 0.5M NaF, 0.3 ml, 0.1M MgCl₂, 0.3 ml, 0.25M cysteine, 0.3 ml, 0.12M NaHCO₃, 1.4 ml, ATP (4.0 mg P_{7 min}/ml), 1.0 ml, and, where added, galactose or tagatose (20 mg/ml) or galactose 6 phosphate (1.88 mg P/ml), 0.8 ml. Side bulb extract, 0.5 ml, 0.5M-NaF, 0.1 ml, 0.12M-NaHCO₃, 0.2 ml. Gas phase 95% N₂+5% CO₂, temp 25° CO₂ output was followed until complete (210 min), and the phosphate esters formed were then separated into soluble and insoluble barium salts and analyzed.)

	Initial P		Soluble Ba salts				Insoluble Ba salts			
	Control galactose or tagatose (mg P)		Galactose-6-phosphate (mg P)				Galactose-6-phosphate (mg P)			
	Control (mg P)	Galactose-6-phosphate (mg P)	Control (mg P)	Galactose-6-phosphate (mg P)	Tagatose (mg P)	Control (mg P)	Galactose-6-phosphate (mg P)	Tagatose (mg P)	Control (mg P)	Galactose-6-phosphate (mg P)
Ortho P	0.00	0.00	0.00	0.00	0.00	0.23	0.24	0.23	0.21	0.21
Acid labile P	3.92	3.92	0.14	1.52	0.16	3.19	1.60	3.23	3.07	3.07
Change in acid-labile P*	—	—	—	+1.38	+0.02	—	-1.59	+0.04	-0.12	-0.12
Acid stable P	2.15	3.65	0.25	0.29	1.70	1.45	1.37	1.55	1.50	1.50
Change in acid-stable P*	—	+1.50	—	+0.04	+1.45	—	-0.08	+0.10	+0.05	+0.05
CO ₂ in 210 min (μl)	—	—	210	726	214	221	—	—	—	—

* Compared with control

The production of acid stable phosphoric esters from galactose

It was found that if precautions were taken to keep the temperature down to 0° during the breakdown of the yeast cells and the purification of the enzyme, there was occasionally obtained from galactose an acid-stable ester which appears in the soluble barium salts. Typical results for the distribution of P with such an extract are given in Table 2, Exp. B. While the phosphorylation of glucose results in the formation of an acid stable ester (2.10 mg P), that of galactose gave a mixture of an acid-labile ester (1.01 mg P) and an acid-stable ester (0.81 mg P). It seems that if these precautions about cooling are taken, a second adaptive enzyme, which is destroyed during the more drastic procedure, is present and forms an acid-stable ester from galactose-1-phosphate.

Using an extract prepared with particular attention to adequate cooling, an attempt was made to isolate the acid-stable ester formed from galactose. The preparation was set up in the same way as for the isolation of α-galactose 1-phosphate described above, but using half the quantities. After 4 hr, 210 mg of galactose had disappeared.

The soluble Ba salts were isolated as described previously: wt 907 mg, P, 5.9%, of which 24.9% was acid labile and 75.1% acid stable. Differential fermentation of the sugar produced by hydrolysis in N-HCl at 100° for 7 min showed it to consist of 86% 'galactose' and 14% 'glucose', and, therefore, the acid labile ester was probably chiefly galactose-1-phosphate. The Ba salt (500 mg ≡ 22.2 mg acid stable P) was converted into the Na salt (120 mg), and 15 ml 5N HCl added. The acidified solution was boiled for 7 min to hydrolyze the acid labile phosphoric esters present, cooled to room

temp, the pH brought back to 8.4 with 10% NaOH, and 3 ml 25% barium acetate added. The precipitate was centrifuged, washed with 10 ml water, and 2 vol 96% ethanol were added to the combined supernatants. The precipitate was centrifuged, washed with ethanol and ether, and dried over CaCl₂ *in vacuo*. The salt was redissolved in 25 ml water, the insoluble material being spun off, reprecipitated with 2 vol ethanol, washed with ethanol and ether, and dried over CaCl₂ *in vacuo*, wt 212 mg, P, 6.18%, acid labile P, nil (Recovery of acid stable P = 59%).

The pentose and ketose in the precipitate were negligible, and the total N was less than 0.1%. The reducing value of the salt, estimated by a Somogyi copper reagent standardized against glucose 6-phosphate, was equivalent to a content of 59.5% barium glucose 6-phosphate (corresponding to 78.2% of the total acid stable P). No insoluble methylphenylhydrazones could be obtained from the barium-free esters, showing that galactose 6-phosphate was not a constituent (Grant, 1935).

The ester was hydrolyzed by a non-specific phosphatase prepared from a dialyzed extract of homogenized rat intestine. A solution of the Na salt (10 ml containing 3.24 mg P) was incubated for 2 hr at 37° with 5 ml extract, 25 ml acetate buffer (pH 5.5), and 1 ml 0.1M-MgCl₂, the inorganic P liberated corresponded to 89.5% of the total P and the P:sugar ratio was 1.00:0.705. For comparison, a similar mixture was set up using sodium glucose 6-phosphate (prepared from 50 mg anhydrous barium glucose 6-phosphate) in place of the unknown Na salt, the inorganic phosphate liberated was 84% of the total, and the P:sugar ratio was 0.91:1.00.

These figures, together with those given in the previous paragraph, suggest that the unknown salt was a mixture of acid-stable esters, one of which is non-reducing and produces a non-reducing substance on hydrolysis, and the other is reducing and contains a reducing sugar. It has not yet been possible to

investigate these esters more completely, but it seems reasonable to suppose that the reducing ester is an aldose-6-phosphate, probably glucose-6 phosphate. The non-reducing ester may be trehalose monophosphate (Robison & Morgan, 1930, Grant, 1935).

The formation of galactose 1-phosphate during adaptation to galactose fermentation

Reiner (1947) investigated the change in acid-labile P during the period immediately preceding the onset of adaptation to galactose fermentation by *Saccharomyces cerevisiae*, and concluded that fermentation was preceded by the accumulation of ATP. He did not separate the phosphoric esters into soluble and insoluble barium salts, however, and in view of the findings presented in this paper, his results might equally well have been explained by the accumulation of galactose-1 phosphate in the prefermentative period, thus stimulating the formation of a second adaptive enzyme attacking galactose-1-phosphate with the production of an acid stable phosphoric ester. This point was investigated by the author using a separation into soluble and insoluble barium salts to differentiate between ATP and galactose-1 phosphate.

(fermentation), there was no accumulation of P in the fraction ($P_{100} - P_{\text{alkali labile}}$) which would have contained galactose-1-phosphate.

DISCUSSION

From the work described in this paper, it seems reasonably certain that the first step in galactose fermentation by Dutch top yeast is the transference of the terminal phosphate group from ATP to galactose to give galactose-1-phosphate. The enzyme catalyzing this reaction is activated by Mg^{++} and by cysteine, and clearly belongs to the class of phosphokinases. It is proposed to call it galactokinase.

By analogy with hexokinase it might be expected that galactose-6-phosphate would be the primary product, but this seems very unlikely for the following reasons: (1) no yeast extract yet investigated can ferment galactose-6 phosphate (Hvistendahl, 1932, Grant, 1935), (2) the enzyme system which forms galactose-1-phosphate from galactose is unable to attack galactose 6 phosphate (Table 4), (3) there is no initial formation of an acid stable ester during the phosphorylation of galactose by the extract (Fig. 2), (4) the extract is unable to phosphorylate tagatose, whereas yeast hexokinase will phosphorylate fructose and mannose in addition to glucose.

It has been shown by Spiegelman, Reiner & Morgan (1947) that the acquisition of the ability to ferment galactose by *Saccharomyces cerevisiae* involves the formation of one or more apoenzymes. The work described in this present paper shows that one of these enzymes is galactokinase.

Since galactose is fermented with the formation of fructose-1,6 diphosphate (Grant, 1935), the question arises as to how the galactose-1 phosphate is transformed into hexosediphosphate. The answer to this question is still obscure, but some indication is given by the work of Kosterlitz (1943b), and by the experiments described in this paper in which a proportion of the phosphate esterified was present as an acid-stable phosphoric ester with a soluble barium salt. If, as seems probable, this proves to be glucose 6 phosphate, then it seems reasonable to suppose that the transformation of galactose-1-phosphate to fructose 1,6 phosphate takes place via glucose 6 phosphate or an ester in equilibrium with glucose-6 phosphate in the system described.

SUMMARY

1. Dutch top yeast has been shown to produce adaptively an enzyme, named galactokinase, which catalyzes the phosphorylation of galactose by adenosine triphosphate forming an ester which has been identified as α galactose-1-phosphate. The enzyme requires Mg^{++} and cysteine for full activity.

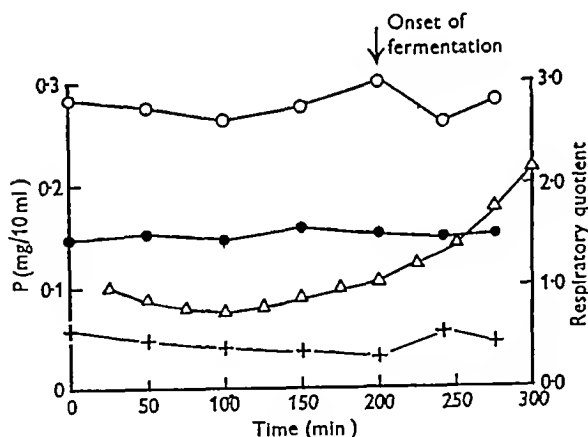


Fig. 3. Distribution of phosphoric esters during the adaptation of Dutch top yeast to the fermentation of galactose. O—O, phosphorus hydrolyzable in 7 min in the insoluble barium salt fraction, ●—●, orthophosphate, +—+, phosphorus hydrolyzable in 100 min—alkali labile phosphorus in the soluble barium salt fraction, Δ—Δ, respiratory quotient.

The separation was carried out according to the method of Umbreit *et al.* (1945), and inorganic phosphate was estimated by the method of Berenblum & Chain (1938). The remainder of the experiment was carried out as described by Reiner (1947), but using Dutch top yeast and incubating at 25°. The results are shown in Fig. 3. It can be seen that, in the period immediately preceding adaptation

2 Galactose-6 phosphate is not an intermediate in this reaction

3 The formation of an acid-stable ester from galactose has been observed occasionally with extracts of galactose adapted yeast

4 There is no evidence for the accumulation of galactose 1-phosphate during the pre adaptive period

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Studies on the Lens

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1 POST-MORTEM CHANGES IN THE MINERAL CONTENT

Our knowledge of the composition and metabolism of the lens has been gained almost exclusively from work on the excised organ, and it is therefore of first importance that post-mortem changes should be fully understood. It might be thought that the use of normal aqueous humour as a bathing medium for the excised lens would make such a study supererogatory, but the results described below show that, even when the lens is left undisturbed in the dead eye, important changes in its mineral content (characteristic of those taking place in a dying tissue) occur within a few hours.

EXPERIMENTAL

To minimize variance due to age, adult rabbits were used in all the experiments described with this species. Except where otherwise stated, the sheep eyes were received from the slaughterhouse within approximately 2 hr of death.

After removing the aqueous humour and cutting away the posterior half of the eye the lens was removed by placing the anterior half of the eye on a flat dish, cornea downwards, reflecting back the curved scleral wall and cutting the suspensory ligament. Before being weighed, the lens was blotted on filter paper. For analysis, the proteins were precipitated by the Somogyi (1930) method and the filtrate made up to a definite volume. Chloride was estimated by the AgIO_3 method of Sendroy (1937). When radioactive isotopes were used, activity was measured in the filtrate on a standard Geiger counter apparatus with the counter described by Maurice (1948). Radioactive sodium (^{24}Na) in the form of NaCl , and bromine (^{82}Br) in the form of NH_4Br were injected intravenously in isotonic solutions, without anaesthesia. After the experimental period the animal was killed by a blow on the neck, and the eyes, together with blood samples, were taken immediately.

For the *in vitro* study of the 'diffusion out' of chloride from the lens, the latter was placed in isotonic Na_2SO_4 solution, while for the 'diffusion in' of sucrose, aqueous humour was diluted with 0.3 vol of an isotonic sucrose solution. Sucrose was determined by hydrolyzing the Somogyi filtrate of the macerated lens with dilute H_2SO_4 , and estimating the reducing value by the Hagedorn-Jensen method.

RESULTS

As a criterion for the normal condition of the lens the chloride content was first chosen. Preliminary experiments revealed that the chloride contents of the lenses of a pair of eyes removed immediately after death did not differ significantly, thus, the mean of the chloride contents of ten left sheep eyes was 43.8 ± 0.2 whilst that for the right eyes was 43.7 ± 0.2 . In the first study, therefore, the two eyes were excised from the freshly killed animal, the lens of one was submitted to analysis immediately, whilst that of the other was analyzed after it had remained in the excised eye for a period of some hours. In Table 1 the chloride contents of the pairs of eyes are

Table 1 *Post mortem changes in the chloride content of lenses allowed to remain in the excised eye*

Species	Time test lens left in eye (hr)	Temp (°)	Chloride content (mg/100 g)	
			Control lens	Test lens
Rabbit	2	0	31.9	31.0
	2	0	32.1	33.7
	4	0	30.7	37.3
	4	0	29.9	35.1
	5	0	33.3	37.1
	4.5	0	31.0	33.9
	6.5	0	25.6	31.0
	7.0	0	36.4	39.3
	5.25	20	33.6	46.3
	Mean		34.0 ± 0.79	39.5 ± 1.14
Sheep	6.0	20	39.4	45.9
	6.0	20	33.1	43.9
	3.0	20	35.6	37.6
	3.0	20	38.2	43.0
	Mean		36.6 ± 0.64	42.6 ± 1.0

shown. The results indicate that the lens left in the eye takes up chloride, thus, for rabbits, the mean chloride content of the control eyes was 34.0 mg/100 g whilst that of the eyes left for more than 2 hr was 39.5 mg/100 g, it will be noted that the lenses left for only 2 hr at 0° showed no increase in chloride content. There is thus a post-mortem increase in the chloride content of the lens even when it is allowed to remain in the eye. It is generally assumed that the chloride content of a normal tissue is a measure of the extracellular space, the cells being virtually free from this ion, the increase in chloride content observed may, therefore, be interpreted as the result either of an increase in extracellular space or of degenerative changes in the lens fibres which permit them to accumulate chloride. Changes in the chloride content should be paralleled by changes in the sodium content on the basis of either of these explanations. Consequently, the uptake of radioactive sodium by the lens *in vivo* should be less than the uptake of another, excised, lens from an artificial medium. Similarly, by use of the radioactive

bromide ion, differing uptakes should be obtained under the two conditions. In Table 2 the results of diffusion experiments are shown, they indicate that when diffusion takes place from an artificial medium

Table 2 *Ratio of the concentrations of isotopes in lens and in aqueous humour in vitro and in vivo studies*

(For *in vitro* studies the lens was suspended in aqueous humour containing the isotope. For *in vivo* studies the isotope was injected intravenously into the animal without anaesthesia. The results are calculated from counts/100 g of material and are expressed as mean \pm s.e. The number of experiments is given in brackets.)

Type of exp	Diffusion time (hr)	Mean ratio isotope in lens/isotope in aqueous humour
<i>In vitro</i> , ^{24}Na	24	0.29 ± 0.01 (6)
	48	0.36 ± 0.07 (6)
<i>In vitro</i> , ^{82}Br	24	0.29 ± 0.03 (6)
	40	0.29 ± 0.03 (6)
<i>In vivo</i> , ^{24}Na	2	0.03 ± 0.02 (6)
	24	0.06 ± 0.01 (4)
	41-46	0.05 ± 0.02 (6)
<i>In vivo</i> , ^{82}Br	24-48	0.07 ± 0.02 (4)

over a period of 24-48 hr at 0° the ratio Na in lens/Na in aqueous humour is 0.29-0.36, whereas *in vivo* experiments gave very much smaller ratios in the region of 0.07 when the diffusion time was either 24 or 48 hr. Similarly, results with the radioactive bromide ion gave large *in vitro* ratios in the region of 0.29 and *in vivo* values of approximately 0.065.

DISCUSSION

The primary importance of the experimental findings described here must be in emphasizing the limitation of *in vitro* experiments on the lens under the given conditions. In some of the experiments described the lens was merely left in the excised eye, i.e. it was surrounded by intraocular fluid during the whole time, and yet quite unmistakable changes in its chloride content took place in a few hours. In the living eye there is strong reason to believe that the aqueous humour flows continuously, being drained away at the angle of the anterior chamber to be replaced by new fluid presumably from the ciliary body, the cessation of this renewal is apparently sufficient, even with a tissue of such low metabolic activity as the lens, to cause changes in the salt content of the lens that are generally interpreted as degenerative, i.e. a breakdown of the normal permeability of the fibre cells to sodium. It is interesting that in a recent publication Palm (1948), working on the penetration of radioactive phosphorus into the lens, came to the conclusion that experiments not completed within 2 hr of the death of the animal were altogether unreproducible.

Although much experimental work has been done on the lens and isolated capsule there seem to be few detailed investigations of the speed of post-mortem changes. On the other hand, the literature is well stocked with references to the changes in composition that occur with development of cataract, changes indicating breakdown of the normal ion permeability of the lens fibres to the sodium chloride in their environment. It is not yet possible to correlate this increase in permeability with specific metabolic changes, but consideration of the following is of interest. Experimental work of the authors (unpublished) supports the results in the literature (Bellows, 1944) indicating that there is no significant decrease of carbohydrate in the lens or aqueous humour during the first few hours following death. The deduction that the presence of glucose does not delay the degenerative changes is supported by the inability of a Krebs-Ringer phosphate medium containing glucose, or an aqueous medium to which isotonic glucose solution has been added, to retard the speed of these changes (unpublished observations).

Much experimental evidence has led to general agreement that the energy required by the normal lens is obtained primarily from carbohydrate metabolism (Bellows, 1944, Fischer, 1930), and further that, unlike other active tissues, the lens has no carbohydrate reserve in the form of glycogen (Weekers & Sullman, 1938).

If we accept these conclusions it is apparent that, following death, a factor is introduced which leads to interference in the utilization of sugar. The importance of an active endergonic metabolism in the normal tissue is further supported by the slowing of the rate of post-mortem changes following a decrease in the temperature of the lens.

2 ESTIMATION OF EXTRACELLULAR SPACE OF LENS

The extracellular volume of muscle has been calculated from its sodium and chloride contents on the well-supported assumption that these ions exist mainly or totally outside the cell, additional methods have involved the study of the uptake of a large water soluble molecule like that of sucrose which is presumed not to penetrate the cell membrane.

The chloride contents of the rabbit and sheep lens were found by analysis to be 34.7 ± 0.6 mg/100 g (10) and 43.7 ± 0.6 mg/100 g (20) (numbers in brackets refer to numbers of experiments). The aqueous humour concentrations of the same eyes, expressed as mg/100 ml, were 397 for the rabbit (6) and 460 for the sheep (14). Consequently the chloride space, i.e. the fraction of the weight occupied by interstitial fluid, assuming that all the chloride is present in the

latter, is 9.5% for the sheep and 8.7% for the rabbit. In view of the unique growth characteristics of the lens, however, it is doubtful whether this fraction does indeed represent the true extracellular space, thus, the lens continues cell proliferation throughout life, the newly formed surface cells displacing the older ones inwards towards the centre, a process that results in the formation of a hard nucleus of old, densely packed fibres and a softer cortex of more recently formed elements. As a result of this development it is quite possible that the fibres of the densely packed nucleus, representing very old cells, contain appreciable amounts of sodium and chloride. If this is true the estimate of the extracellular space from the chloride content must give too high a value. Moreover, the cells containing sodium and chloride will be predominantly in the dense highly viscous nucleus, and will be therefore unable to attain diffusion equilibrium with their environment within an experimentally measurable period. In the present work the fraction of the lens that comes into apparent diffusion equilibrium, in respect to chloride, ^{24}Na , ^{82}Br and sucrose with its environment within 24–48 hr, has been determined. We may call this the 'readily available space'. In so far as the chloride ion was concerned, the space was estimated on the basis of the amount that diffused out of the lens into an isotonic sulphate solution within 24–48 hr. For ^{24}Na the results already described on the diffusion into the lens *in vivo* were used for the computation. Sucrose was allowed to diffuse into the excised lens from an aqueous humour medium, in view of the post mortem changes already described we may expect this substance to provide altogether too high a value for the 'readily available space'. This was indeed found to be the case, washing the lens, after the diffusion in sucrose, for 0.5 hr in a sulphate medium reduced the 'sucrose space' to a value comparable with the 'readily available chloride space'.

RESULTS

The results of this investigation are summarized in Table 3. It will be seen that the 'readily available chloride space', determined by the fraction of chloride that diffuses out of the lens in 24–48 hr, is 5.5% as opposed to the value of 8.5–9.6% for the total chloride space. On the basis of *in vivo* diffusion of radioactive isotopes into the lens the 'readily available space' is 6.1–6.7%. Sucrose diffuses into an available space of 13.5%, indicating the occurrence of post mortem changes. If the lenses, after the diffusion of sucrose into them, are placed in a sucrose-free medium, there is an initial rapid diffusion of sucrose out, this rapid loss is succeeded by a much slower loss during the next 24 hr. If the sucrose space is computed on the basis of the sucrose content after this rapid loss it averages 5.5%.

Table 3 *Values of the extracellular volume of lens*

Method	Total space (%)	Readily available space (%)
Chloride analysis and diffusion out into Na_2SO_4	9.6 (sheep) 8.5 (rabbits)	5.5 (sheep) —
Diffusion of ^{24}Na <i>in vivo</i>	—	6.1 (rabbits)
Sucrose (diffusion into lens)	13.5 (sheep)	—
Sucrose (diffusion into lens and subsequent washing in isotonic Na_2SO_4)	—	5.46 (sheep)
Diffusion of ^{82}Br <i>in vivo</i>	—	6.7 (rabbits)

DISCUSSION

For kinetic studies on the penetration of substances from aqueous humour into the lens fibres a knowledge of the magnitude of the effective extracellular space—the 'readily available space' discussed here—is a first essential. The results described in this paper indicate that this space is of the order of 6–7% where *in vivo* studies are concerned. Owing to its greater density, the nucleus of the adult lens contains a higher concentration of potassium (expressed in mg/unit wt. of lens) than the cortex, the ratio $[\text{K}]/[\text{Na}]$ in the nucleus is, however, lower than that in the cortex (Bellows, 1944). If it is assumed that sodium is predominantly an extracellular ion, it might be deduced that the nucleus has a greater extracellular matrix. This conclusion appears doubtful on histological grounds since the hard,

viscous, nucleus is composed of densely packed fibres. A more probable explanation for the lower ratio is that the nuclear fibres are partly permeable to sodium, thereby permitting sodium and chloride to displace potassium, an explanation with which the present findings are in agreement. It is apparent that the nucleus must have a very low metabolism indeed and this fact, coupled with the disappearance of the cell nuclei from these central cells, would support the view of an increased permeability to sodium.

SUMMARY

1 The limitation of the *in vitro* experiment on the excised lens is deduced from studies of the rate at which post-mortem changes occur.

2 Three methods for the determination of the 'readily available space' are described.

3 Analysis of the results shows that this space is 5.0–5.5% of the volume of an adult sheep's lens and approximately 6% of the volume of an adult rabbit's lens.

4 From an analysis of the K/Na ratio in the nucleus and the cortex, together with anatomical and histological evidence, it is suggested that the chloride that fails to diffuse out of the lens within 24–48 hr. is contained in the nucleus.

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Breakdown of Cozymase by a System from Nervous Tissue

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Cozymase has been observed to be unstable in many biological systems (Harden & Young, 1906a, b; Schlenk, 1945; McIlwain, 1947; McIlwain & Hughes, 1948). In brain tissue Mann & Quastel (1941) and Handler & Klein (1942) showed the loss of cozymase to be due to a system which liberated nicotinamide from the coenzyme, and which was inhibited by added nicotinamide. Since then many investigators have taken precautions to prevent the breakdown when using systems dependent on cozymase, but the

breakdown has not been studied sufficiently for an assessment to be made of its significance in the normal activities of nervous tissue. Because of the important part played by cozymase itself, we considered it especially necessary to determine the speed of the breakdown, and its variation with cozymase and nicotinamide concentrations and with other factors likely to affect it in the living organism. The breakdown has been found to be very rapid, and to involve the formation of acid (see equation I, p. 474).

EXPERIMENTAL

Materials

Cozymase specimens Pure cozymase has not been obtained, but the reaction has been carried out with three different specimens of the substance, for which analytical data are given in Table 1. Specimen D was used in the majority of experiments. Specimen A, Table 1, is specimen A of Table 1 of Mellhain & Hughes (1948), and further data concerning it are given in that paper. The determinations of catalytic activity quoted in Table 1 were made in the apozy-masesystem under conditions previously described (Mellhain & Hughes, 1948). The nicotinamide contents quoted for specimens D and E were obtained by liberating the amide by excess of the brain enzyme, and determining the nicotinamide colorimetrically (see below).

Table 1 Analytical data concerning cozymase specimens

Determination and standard value	Cozymase specimen		
	A	D	E
I Acid formation in reaction with $\text{Na}_2\text{S}_2\text{O}_4$ (Warburg & Christian, 1936) ($\mu\text{mol}/\text{mg}$)	1.75	2.70	2.58
Purity by comparison with theoretical value of $4.53 \mu\text{mol}/\text{mg}$ ($\text{C}_{21}\text{H}_{27}\text{O}_{14}\text{N}_7\text{P}_2$, mol wt 663) (%)	39	61	57
II Nicotinamide content (%) (theoretical value 18.6)	8.0	11.5	9.9
Purity (%)	43	62	53
III Catalytic activity in apozy-mase system ($\mu\text{mol CO}_2/\mu\text{g}/\text{hr}$)	1.47	2.40	—
Purity (by comparison with value of 4.0 derived from Axelrod & Elvehjem, 1939 and Mellhain & Hughes, 1948) (%)	37	60	—
Origin of specimens	This laboratory	Schwartz Laboratories, Inc	

Tissue preparations Guinea pigs, rabbits and rats were killed by a blow on the neck, sometimes bled, the skull opened and the brain put on ice, or ground, within 3–4 min of death. Sheep brain and cord were obtained from a slaughterhouse as soon as possible after killing and brought to the laboratory in ice water. Tissue was ground by hand with an equal weight of acid washed sand in a cold mortar or homogenized in the glass apparatus of Potter (see Umbreit, Burris & Stauffer, 1945). Crushed tissue was prepared by pressing a fragment approx $3 \times 3 \times 2 \text{ mm}$ to approx 0.3 mm thickness between slides, it was then handled as a slice. A teased preparation of white matter was made by drawing a fine needle through the material in the direction of the fibres.

Ground preparations with which several experiments were to be performed were prepared as follows (cf Handler & Klem, 1942). Tissue (25 g) and sand (25 g) were rubbed for

5 min in a mortar and the tissue washed into centrifuge tubes with 100 ml of 0.9% NaCl, leaving behind the bulk of the sand. After centrifuging, the supernatant was discarded and the precipitate suspended in a further 100 ml of NaCl. In most instances reported here such washing was performed six times in all, although the preparations did not noticeably change after one or two washings (see p 472). Preparations were stored at $2-4^\circ$ as suspensions containing tissue from 1 g fresh weight of brain in 2 ml of 0.9% NaCl saturated with toluene. Metabolic quotients were calculated from the dry weights of the whole tissue from which the preparations had been made.

Determination of nicotinic acid and nicotinamide

The reaction with cyanogen bromide (see especially Teeri & Shimer, 1944) has been adapted to the special problems associated with the determination of nicotinamide in the presence of cozymase.

Reagents The phosphato buffer was that of Martinek, Kireh & Webster (1943). CNBr solution was prepared from sat. Br_2 water (500 ml) and KCN (10% w/v, 70–80 ml) at $5-10^\circ$ and kept in a refrigerator for not more than a week. The necessary concentration of HCl used was found to be approx 5N for nicotinic acid and N for nicotinamide.

Nicotinic acid To the CNBr (2.5 ml) and buffer (5 ml) solutions in glass stoppered tubes, the specimens (containing up to $0.5 \mu\text{mol}$ nicotinic acid in 5 ml) were added and the mixtures left at room temperature for 35 min, *m*-phenylenediamine dihydrochloride (5% in HCl, 0.5 ml) and HCl (0.5 ml 5N) were added rapidly, mixing after each addition. The intensities of the resulting yellow colours were determined within 30 min in a Spekker absorptiometer with Ilford spectrum filter no 601. A reagent blank (water substituted for the test solution) was subtracted.

Nicotinamide The amide yielded about a quarter of the colour intensity afforded by the acid under the above conditions (cf Melnick & Field, 1940; Perlzweig, 1947). Several workers have used heat during the reaction with CNBr to reduce the reaction time and increase the colour intensity (Bandier & Hald, 1939; Martinek *et al* 1943), in the presence of cozymase we found most satisfactory the following modification of the method described above for nicotinic acid. The mixtures of CNBr, buffer and specimens (up to $0.5 \mu\text{mol}$ nicotinamide) were kept at 37° for 30 min, cooled to approx 10° , the amine (0.5 ml) and HCl (0.5 ml N) added, and intensities determined within 40 min.

Preparation of specimens for analysis Specimens were mainly from reaction mixtures containing brain tissue or preparations, and it was necessary to deproteinize them without liberating nicotinamide from cozymase. $\text{Zn}(\text{OH})_2$ was found satisfactory. To the specimen (containing approx 5–40 mg wet wt of tissue) in a graduated centrifuge tube ZnSO_4 (8% w/v, 0.2–0.4 ml) was added and the tube put in ice water. NaOH solution, equal in volume to the ZnSO_4 , and of a concentration previously found sufficient to remove the Zn and leave a neutral solution, was added followed by water to 7 or 10 ml. The tubes were centrifuged and samples taken for nicotinamide determination.

Acid formation during cozymase breakdown

In the main compartments of Warburg vessels (conical, of approx 15 ml) were placed 2 ml of a solution containing NaHCO_3 (0.009M) and NaCl (0.9%), freshly gassed with

5% (v/v) CO_2 N_2 . To the side arms were next added 0.5 ml of the bicarbonate saline (controls) or cozymase freshly dissolved in bicarbonate saline (experimental vessels). The tissue suspension (0.5 ml, in 0.9% NaCl) was then added to the main compartments, yellow P to the centre cups, the vessels fitted to manometers and equilibrated with the CO_2 - N_2 at 37° . Successive readings were usually steady 15–20 min after placing in the thermostat, the contents were then mixed and readings taken at 3 or 5 min intervals.

Other physiological salines used were those of Krebs & Eggleston (1940).

RESULTS

Rate of reaction

Estimation by nicotinamide formation First indications of the high reaction velocity were obtained by following the course of liberation of nicotinamide by brain tissue ground with sand (Fig. 1). Cozymase concentrations were employed which approximated to those of the pyridine nucleotide content of animal tissues. After stopping the reaction and removing protein (both accomplished by zinc hydroxide precipitation) nicotinamide was determined by the Koenig reaction. The initial velocity in Fig. 1 corresponds to the decomposition of $1.8 \mu\text{mol}$ of cozymase/mg dry wt of whole tissue/hr. Other experiments by this method under conditions similar to those of Fig. 1 gave quotients of 1.0–1.4 with tissue from different species (Table 2).

Direct comparison between the rate of breakdown of cozymase and the rate of respiration was made with material from guinea pig brain. Slices (mixed grey and white matter from cerebral hemispheres)

absorbed 0.64 , $0.60 \mu\text{mol O}_2/\text{mg dry wt/hr}$ in glucose saline, without Ca salts, at pH 7.4 and at 37° (O_2 as gas phase). Values in saline with Ca (0.005M) were 0.4 , $0.38 \mu\text{mol O}_2/\text{mg dry wt/hr}$. These are

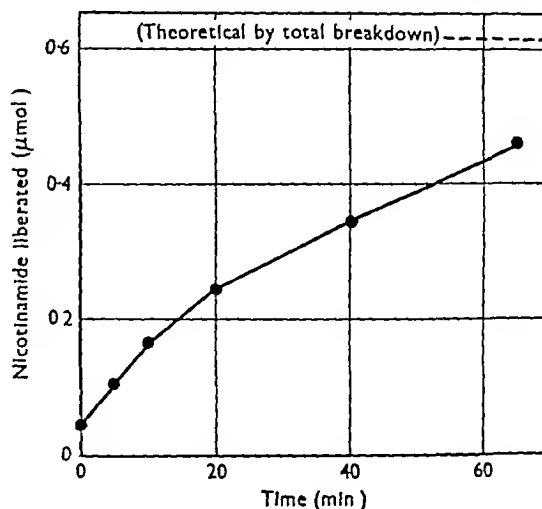


Fig. 1. Course of liberation of nicotinamide from cozymase ($0.62 \mu\text{mol}$) by ground rabbit brain (0.4 mg dry wt) in 3.2 ml phosphate Ringer at pH 7.4, 37° , aerobic.

typical values (Warburg, 1924, Quastel, 1939, Ellott & Libet, 1942). Ground tissue from the same hemispheres decomposed 1.25 , $1.35 \mu\text{mol cozymase/mg dry wt/hr}$.

Activity of different tissue preparations The rate of reaction increased with the degree of damage of the tissue (Table 2), being less in slices or crushed tissue

Table 2. Cozymase breakdown by different species and preparations

Animal	Tissue	Treatment	Rate of breakdown ($\mu\text{mol/mg dry wt/hr}$)
Rabbit	Whole brain	Ground with sand	1.4
Guinea pig A	"	"	1.0, 1.4
Sheep A	"	"	1.3
Mouse	"	"	0.5
Sheep B	Cord	Homogenized	1.08
	White matter from corpus callosum	"	0.86
	Grey matter from lateral convexity of hemispheres	"	0.36
	Grey matter adjoining longitudinal fissure	"	0.36
Guinea pig B	Whole cerebral hemisphere	Ground with sand, not washed	1.02
	"	Ground with sand, washed 3 times	1.03
	"	Homogenized in equal weight of saline	1.15
	"	Homogenized in $\times 20$ wt of saline	1.12
Guinea pig C	Largely white matter from centrum ovalae	Sliced	0.28, 0.46
	Largely grey matter from cerebral cortex	"	0.13
Guinea pig D	Largely corpus callosum	Teased	0.43
	Mixed tissue from cerebral hemispheres	Crushed	0.51
	" " " "	Homogenized	1.56

than in homogenates or material ground with sand. In grinding with sand, little difference was found between a preparation rubbed for 0.5 and one rubbed for 5 min. No appreciable difference was found between the activity of a suspension of guinea pig brain made 2–3 min after death, and another made after the excised tissue had been left at room temperature for 30 min. White matter was more active than grey (Table 2).

Acid formation during the reaction

The findings of Handler & Klein (1942) on the breakdown of cozymase suggested to us that the end products might be more acidic than cozymase, since the reaction involves the conversion of a pyridinium salt, the salt of a strong base, to a much more weakly basic pyridine derivative (see equation I, p. 474). The breakdown of cozymase has been followed in previous work either by measuring the fall in catalytic activity of cozymase containing solutions or by measuring the nicotinamide produced. Measurement by acid formation would be much more convenient in studying the reaction kinetically, but it was first necessary to find whether the same breakdown was in fact being observed. Relevant findings are given below.

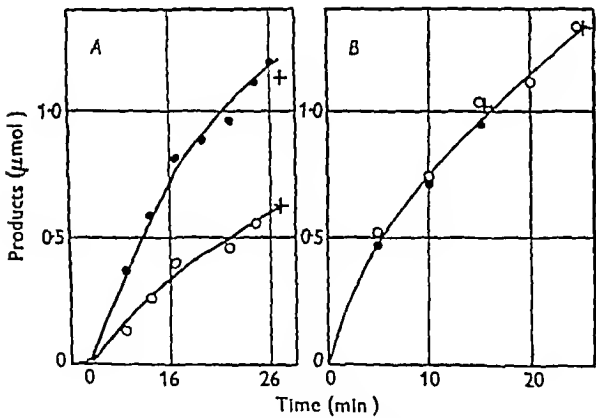


Fig. 2. Course of evolution of CO_2 from NaHCO_3 containing solutions during breakdown of cozymase by ground brain (points), relationship to nicotinamide released (crosses) (A) Rabbit, pH, 7.3, maintained by NaHCO_3 (0.021 M) and $\text{CO}_2\text{-N}_2$ (5% v/v). Reaction mixtures contained also 0.9% NaCl, 1.24 μmol cozymase and the following dry weights of tissue: $\text{—}\circ\text{—}\circ\text{—}$, 1 mg; $\text{—}\bullet\text{—}\bullet\text{—}$, 2.5 mg. (B) Sheep, pH 6.8 (by NaHCO_3 , CO_2), cozymase, 2.4 μmol , 4.2 mg dry weight of tissue, otherwise as (A). The two sets of points (\circ and \bullet) refer to duplicate reaction mixtures. Crosses indicate nicotinamide released at the end of each experiment.

Quantity of acid produced. By carrying out the reaction in bicarbonate-containing solutions in manometric apparatus it was possible to observe a smooth evolution of CO_2 (Fig. 2). The use of such conditions for measuring the rate of the reaction was, therefore,

examined. Anaerobic conditions were generally employed, to minimize the possibility of associated reactions. The brain preparations after three washings with 0.9% sodium chloride caused very little gas change at the low concentrations in which they were used (0.5–2 mg dry wt./ml, 3 ml./vessel). Reaction in the absence of cozymase was relatively very small even when fresh homogenates of tissue were used without any washing. The commonest observation was of gas absorption in the absence of substrate, at a rate between 0 and 10% of the rate of reaction with cozymase. Such changes were allowed for by subtracting the change in a tissue control from those in experimental vessels. Any retention of CO_2 by the preparations was within experimental error. Using such methods, the CO_2 evolved was found to be approximately equivalent to the cozymase decomposed (Fig. 2). Seventeen experiments with five different preparations, including sheep, rabbit and guinea pig brain, gave for the ratio CO_2 evolved/nicotinamide formed, a mean value of 0.98 with standard deviation of 0.08.

With limited quantities of cozymase the gas evolution reached a well-marked end point equivalent to the cozymase added. At this stage the enzyme was not inactive, this was shown by addition of further cozymase. The end point is being approached in one experiment of Fig. 2A.

Inhibition by nicotinamide. Mann & Quastel (1941) found 0.1–0.01 M-nicotinamide prevented the inactivation of cozymase by animal tissues, including brain. This finding has been applied by many subsequent workers in experiments which required

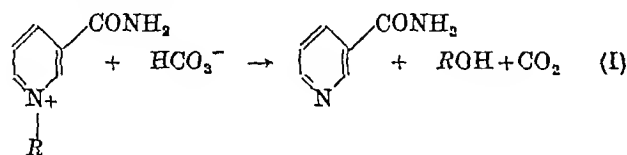
Table 3. Inhibition of the breakdown of cozymase by nicotinamide

(Reaction mixtures contained a sheep brain preparation (1.7 mg dry wt.) and the nicotinamide in 2.5 ml NaCl-NaHCO_3 . Cozymase (in 0.5 ml) was added at zero time, and the reaction run for 26 min. The rates of the last column were determined graphically.)

In reaction mixture		Produced		Initial rate of CO_2 evolution ($\mu\text{mol}/\text{mg}$ dry wt./hr)
Cozymase (μmol)	Nicotinamide (μmol)	Nicotinamide (μmol)	CO_2 (μmol)	
1.24	0	1.05	1.01	1.26
1.24	1.0	0.92	0.96	1.13
1.24	2.0	0.82	0.78	0.75
1.24	4.0	0.60	0.55	0.56
1.24	8.0	—	0.26	0.34

intact cozymase, relatively high concentrations of the amide (e.g. 0.02 M) being employed. We found both 0.01 and 0.002 M-nicotinamide almost completely inhibited the formation of acid from cozymase by washed sheep brain, under the experimental conditions described above. This additional evidence supports the validity of the manometric method for

following cozymase breakdown. Moreover, the reaction was found to be extremely sensitive to nicotinamide. A quantity equimolar to the cozymase content of a typical reaction mixture markedly lowered the reaction rate (Table 3). Because of this sensitivity it was possible to follow the inhibited reaction quantitatively not only by acid formation but also by nicotinamide production. Good agreement was obtained between measurements made in these two ways (Table 3). This makes it probable that the acid and nicotinamide are indeed formed in the same reaction, and, accepting the evidence of Handler & Klein (1942), this may be formulated as in (I), though we have not fully investigated alternative possibilities.



Rate of cozymase breakdown under various conditions

The effect of a variety of circumstances on the breakdown was examined. In doing this the rate of acid formation was largely used, but results were confirmed in each set of experiments by measuring at the end of the reaction the total nicotinamide formed.

pH, salts, oxygen. The reaction showed an optimum pH between 6.5 and 7 (Fig. 3). Most manometric experiments were, therefore, carried out at pH 6.9,

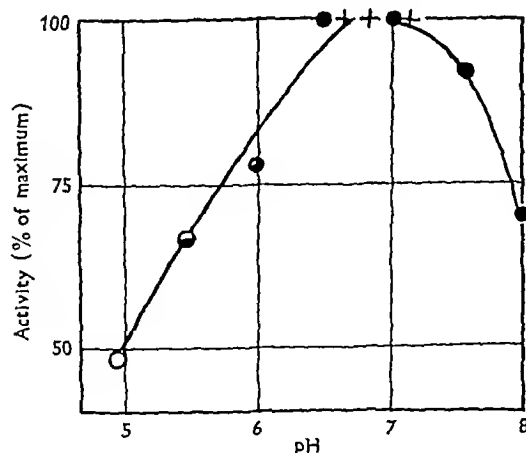


Fig. 3 Change in rate of cozymase breakdown with pH. ●, 0.02M phosphate buffers, guinea pig brain, ○, 0.1M acetate buffers, guinea pig brain, +, bicarbonate 5% v/v CO₂, rabbit brain. Initial concentration of cozymase 2×10^{-4} M.

obtained by 0.0075M-sodium bicarbonate in equilibrium with 5% (v/v) CO₂ N₂. Great variation in the ionic composition of the medium was possible without significant change in the reaction velocity

(Table 4 and Fig. 3). The reaction velocity was compared in air/CO₂ and N₂/CO₂ (with yellow phosphorus to maintain anaerobiosis), and no difference was found.

Table 4 Cozymase breakdown in different media

(All mixtures contained cozymase $(2 \times 10^{-4}$ M) and were at pH 7.4. Each of the velocities quoted was calculated from three determinations of the nicotinamide liberated at different times during the reaction.)

Suspending medium	Rate of breakdown (μmol nicotinamide liberated/mg dry wt./hr.)	
	With guinea pig brain	With rabbit brain
Physiological saline with Ca	1.07	1.26
Physiological saline without Ca	1.17	1.25
Phosphate buffer (0.02M)	0.96	1.17

Stability of preparations. Ground sheep brain, washed and suspended (4.4 mg dry wt./ml) in 0.9% sodium chloride at pH 7.0, retained less than 5% of its activity after heating for 15 min at 100 or 70°. At 60°, 20% of the activity remained and at 50°, 40%. At 37°, no loss occurred in 1 hr at pH values (in phosphate) of 5.6, 6.8 and 8. Preparations were normally stored in a refrigerator at 2–3°, with 0.5 g wet wt. of tissue/ml of 0.9% sodium chloride. Most preparations had been washed with 0.9% sodium chloride (volume, five times the bulk of fresh tissue) by centrifuging either three or six times, and were, without deliberate adjustment of pH, at pH 7. Toluene was added as a preservative and under these conditions little loss in cozymase splitting activity occurred during 1–2 months. Some loss was found in preparations 3 or 4 months old, and in a more recent preparation in which bacteria had grown.

Variation in velocity with cozymase concentration, different cozymase specimens. Figs. 1 and 2 show that the velocity of breakdown of cozymase falls towards the end of experiments in which cozymase concentration is decreasing, and the concentration of nicotinamide, which inhibits the reaction, is increasing. To differentiate between these two factors, kinetic experiments were carried out with different initial concentrations of cozymase, and initial velocities of breakdown obtained from graphs such as those of Fig. 2. The initial velocity fell relatively little at concentrations down to 6×10^{-4} M, the lowest at which the ordinary manometric method is applicable. The reaction velocity at concentrations below this was, therefore, followed by nicotinamide determinations. The results (Fig. 4) were not sufficiently accurate for determining the equilibrium constants of the system, but showed that the velocity did not fall to half the maximum until the concentration of cozymase was less than 10^{-4} M. A higher value is given by Spaulding & Graham (1947) for unspecified

tissues which may include brain. Rates of breakdown of specimens A, D and E of cozymase (see Table 1) were compared at concentrations between 1.3 and $4 \times 10^{-4} M$, no differences were found.

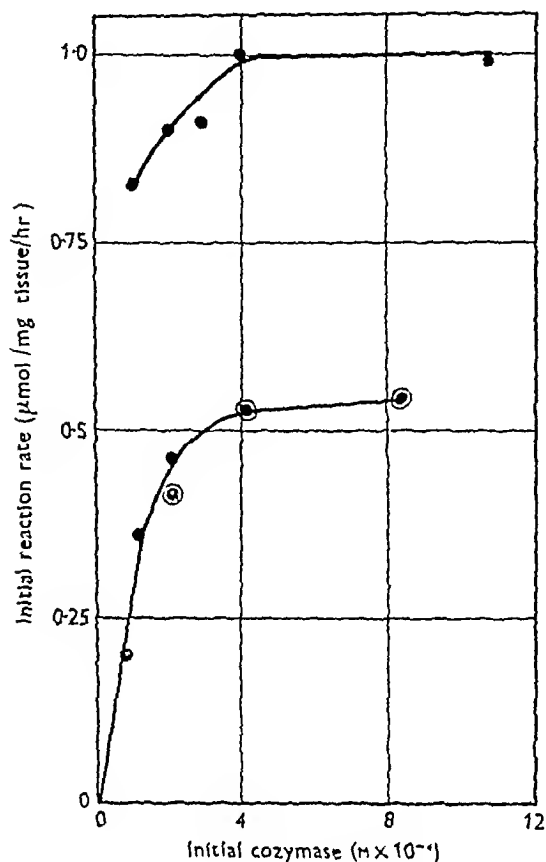


Fig 4 Change in initial rate of cozymase breakdown with cozymase concentration, in two preparations of guinea pig brain. Above cozymase E, below, cozymase D. ○, determined by CO_2 evolution, ●, by nicotinamide formation.

Extraction of cozymase-splitting enzyme

In the preceding experiments a suspension of tissue particles in 0.9% sodium chloride was used in which the activity was firmly associated with the particles (Table 2). The active material readily dissolves in water with little loss, together with a large part of the tissue preparation, giving an opalescent solution, as is shown in Table 5 the activity/unit dry weight is increased in the process. Sheep brain has been extracted similarly, both before and after standing with toluene. The properties and treatment of such extracts will be described later.

DISCUSSION

The following findings must be taken into account in assessing the part played by the breakdown of cozymase in preparations from nervous tissue. (1) Its velocity of 0.4 – $1.8 \mu\text{mol/mg dry wt/hr}$. This is outstanding. The rate of reaction, even on a molar

basis, can be greater than the respiratory rate of the tissue, it can lead to the breakdown of the tissue's own dry weight of cozymase each hour. (2) This velocity is reached at the concentrations of cozymase occurring in the tissue. (3) The reaction is inhibited by nicotinamide, also at concentrations comparable to those at which nicotinamide derivatives occur in the tissue. It is (4) most rapid in damaged tissue, leads (5) to loss of the catalytic activity of cozymase and (6) to the formation of hydrogen ions at an equivalent, high velocity.

Table 5 Extraction of cozymase splitting system from guinea pig brain

Preparation	Activity (by CO_2 evolution at pH 6.8)	
	$\mu\text{mol cozymase split/material derived from 1 g fresh wt/hr}$	$\mu\text{mol cozymase split/mg dry wt of preparation/hr}$
(1) Whole brain ground with sand	224	1.12
(2) Preparation (1) washed with saline and extracted with water (5 ml/g fresh brain), extract	77	1.6
(3) Residue from (2) re-extracted as before, extract	77	1.4
(4) Residue from (3)	58	0.6
Sum of (2), (3) and (4)	212	—

Reaction velocity. Although the reaction has been investigated in ground nervous tissue and has frequently been described as rapid, kinetic experiments leading to dependable values for its rate do not appear to have been reported. An isolated statement is made by Spaulding & Graham (1947, in a footnote to Table 1), but not discussed further, that 'the nucleosidase/g of dry brain split approximately 15 mm of DPN in 20 min'. Interpreting mm as millimol, the velocity corresponds to 100 times that which we find, on the other hand, most other measurements of cozymase in Spaulding & Graham's paper are in micromol quantities, and if mm means micromol the speeds are about 1/10 those which we find. Spaulding & Graham give the relative velocities of breakdown by ground rat brain and heart as in the ratio 100:27. The rate of breakdown of cozymase by guinea pig heart can be derived indirectly from measurements by Govier & Jetter (1948) as about $0.3 \mu\text{mol/mg dry wt/hr}$, ignoring possible species differences, this would suggest a value of about $1.1 \mu\text{mol/mg/hr}$ for brain. This is close to our own findings and appears to be the most satisfactory interpretation of the observations of Spaulding & Graham (1947). We have preferred not to use a name

such as diphosphopyridine nucleotidase for the system, as the specificity of its action has not yet been examined

Dependence on nicotinamide and cozymase concentrations The concentration of cozymase in fresh brain tissue can be calculated from values given by Axelrod, Madden & Elvehjem (1939) as approx $5 \times 10^{-4}M$. Values for total nicotinic acid derivatives are close to this (Mitchell & Isbell, 1942, Taylor, Pollack & Williams, 1942). With such an initial concentration of cozymase, our results show that the breakdown can proceed at over 80 % of its maximum velocity, but that a fall in cozymase concentration results in a fall in the velocity of the reaction (Fig. 4). Also, the reaction is inhibited by comparable concentrations of nicotinamide, one of its products. Therefore, although the operation of this reaction at its maximum velocity would lead to very rapid depletion of the cozymase of the tissue, the velocity of breakdown would be expected to fall considerably as the substrate disappeared and the product accumulated.

Possible operation of the reaction in vivo Although the breakdown of cozymase is particularly rapid in brain tissue, and may there subserve a special purpose, it occurs in many animal tissues (cf. Schlenk, 1945, Spaulding & Graham, 1947, Govier & Jetter, 1948) and also in bacteria (McIlwain & Hughes, 1948). Data are only rarely available for full characterization of the reaction, but it is readily differentiated from that of the nucleotide pyrophosphatase (Kornberg, 1948) which does not liberate free nicotinamide. The process in streptococci liberated nicotinamide, but was not associated with acid formation (McIlwain & Hughes, 1948), there, however, intact cells were being examined, and greater possibility existed for coupling with further changes. The major problem in assessing the physiological importance of the reaction lies in reconciling its high velocity with the undoubted importance of intact cozymase. In general, this suggests that the breakdown may be intermittent, owing to some physiologically controlled inhibition, that the breakdown must be counterbalanced by synthesis, and that the reaction may constitute part of a system controlling cozymase-requiring reactions by regulating the cozymase level.

Considering more specifically mammalian brain, the cozymase content of the fresh organ in a given species has been found to be relatively constant (Axelrod *et al.* 1939), and breakdown of added cozymase is smallest when least damage has been done to the tissue. In the tissue, which of course contains more than one cell type, the process is presumably inhibited for a large part of the time (possibly by

separation of the reactants), and balanced by re-synthesis if and when it takes place. Stability of the native cozymase of the tissue is being examined separately, but it is possible to make the following observations from the present data. The reaction seems suited to physiological operation and control in that its pH optimum is within physiological range, its relation to substrate concentration is such that with the cozymase concentration found in the tissue as a whole the reaction would be rapid initially, but decrease in rate as cozymase is lost and nicotinamide accumulates, the high initial velocity makes it less likely that reaction with cozymase is an incidental property of a system whose main reaction is with another substrate. The phenomena of functional importance in nervous tissue are intermittent, extremely rapid and associated with ion migration and permeability changes. These give many possibilities for the functional importance of a reaction with the characteristics of the cozymase breakdown, but more data are required for their detailed discussion.

SUMMARY

1 The process by which tissue preparations from the central nervous system liberate nicotinamide from cozymase proceeded at rates of 0.4–1.8 $\mu\text{mol/mg dry wt/hr}$, this was often greater than the rate of respiration by the tissue.

2 The reaction approached its maximum velocity at the natural concentration of cozymase in brain tissue ($5 \times 10^{-4}M$), half the maximum velocity was reached at less than $10^{-4}M$ -cozymase.

3 The breakdown was very sensitive to nicotinamide, $10^{-3}M$ -nicotinamide reduced the rate of breakdown of $3 \times 10^{-4}M$ -cozymase to half its uninhibited value.

4 The process resulted in the formation of one equivalent of acid/molecule of nicotinamide liberated. This was apparently due to the disappearance of the pyridinium ion, and when the reaction was carried out in $\text{NaHCO}_3\text{-CO}_2$ buffers its velocity could readily be followed manometrically by CO_2 evolution.

5 The optimum pH of the reaction was approx 7, and the velocity was almost unaffected by many inorganic salts.

6 The system responsible for the reaction remained associated with tissue debris in isotonic solutions, and was fairly stable at 0 or 37°, but inactivated rapidly at 70°. It was easily obtained in solution in water.

We are indebted to Mr J. D. Cheshire for assistance during these experiments.

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The Oxidation of Catechol and Homocatechol by Tyrosinase in the Presence of Amino-acids

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In the early stages of the oxidation of catechol by crude extracts of potato or mushroom the formation of reddish purple pigment can be detected. Attention was drawn to this phenomenon first by Szent-Györgyi (1925), who achieved some degree of separation from potato juice of the substance which, together with enzyme and catechol, was responsible for the pigment formation. He suggested the name 'tyrin' for it and speculated as to its possible significance as a reversibly oxidizable hydrogen carrier. Platt & Wormald (1927), however, subsequently concluded that 'tyrin' was merely a mixture of amino acids, and no more has been heard of it. Although, as Platt & Wormald believed, many amino acids can cause pigment formation when present in solutions in which tyrosinase is oxidizing catechol, there is now reason to think that the amino-acid contributing most strikingly to the phenomenon originally observed is L-proline. The identification of L-proline as a constituent of mushroom extract, and the results of a study of some features of the reaction involved in the production of the pigment, are described in this paper.

METHODS

Tyrosinase preparations These were made from the common mushroom (*Psalliota campestris*) according to the directions of Keilin & Mann (1938), but with the omission

of some of the later steps in the purification described by these workers. Various preparations with Q_{O_2} values ranging between 50,000 and 500,000,* were used in the course of the work. Within these limits variation in the purity of the enzyme preparation has no qualitative effect on the reactions which are to be discussed.

Measurement of oxygen uptake The Warburg manometric apparatus was used. The total volume of reaction mixture was 2.0 ml, the components being distributed in 0.1 M phosphate buffer of pH 7.0. The enzyme was tipped into the substrate solution from the side arm at zero time, and in critical experiments the inset cup of the reaction vessel contained 20% KOH and a roll of filter paper. In the earliest experiments the temperature of the water bath was 20°, but was changed to 25° when the advent of warmer weather made the maintenance of the lower temperature difficult.

Colorimetry In experiments where quantitative assessment of pigment formation was attempted a Spekker photoelectric absorptiometer was used with a 1 cm cell and a green filter (no. 5 of Hilger set H 455, later replaced by Ilford spectrum green no. 604). The destruction of the pigment in acid solution was similarly studied, readings being taken at timed intervals after addition of acid to the pigment solution.

Measurement of pH and hydrogen ion liberation A glass electrode was used for pH measurement. When it was desired to study the hydrogen ion liberation accompanying the oxidation of catechol in the presence of amino acid the reaction was carried out in unbuffered solution at pH 7.0.

* Q_{O_2} values in $\mu\text{l/mg dry wt/hr}$, substrate catechol, determined manometrically according to Keilin & Mann (1938).

A glass electrode was immersed in the reaction mixture, which was aerated with CO_2 free air or O_2 . Standard NaOH was added from a microburette as necessary to maintain the pH at its initial value. The amount of alkali added was then the measure of the hydrogen ion liberated during the reaction.

RESULTS

Preliminary attempts to isolate from mushroom extract the substance responsible for the pigment formation suggested that this substance might be an amino-acid. Of the available pure amino acids tested in the tyrosinase-catechol system, only proline and hydroxyproline gave a colour similar to that given by the mushroom concentrates. That one of these acids might be concerned was further suggested by the fact that the more active the concentrate, the lower was the ratio of amino nitrogen to total nitrogen in it. When *p*-benzoquinone was shaken with proline in ethanol a very similar colour was produced suggesting that the pigment formation in the enzyme reaction was the result of a secondary reaction of amino-acid with *o*-benzoquinone, analogous to anilinoquinone formation in the presence of aniline (Fugh & Raper, 1927).

Isolation of L-proline from mushrooms

The mushrooms were minced into 95% ethanol, and after standing 2-3 hr, with frequent stirring, the supernatant fluid was decanted and the tissue squeezed out in muslin in a hand press. After filtration of the combined fluids the ethanol was removed under reduced pressure and the residue taken up in water. Sufficient basic lead acetate was added to ensure maximum precipitation, and the precipitate was removed by filtration. After removal of the lead by H_2S the filtrate was taken to dryness at 30° *in vacuo* and extracted with cold methanol. The methanol was removed from the extract under reduced pressure, and the residue dissolved in water and precipitated with Reinecke salt. This precipitation was carried out in two stages, separating first the precipitate formed at 25° , and then lowering the temperature to 5° , when a second crop of Reinecke salt was obtained. The precipitates were decomposed separately by the method of Kapfhammer & Eok (1927), and the solutions were evaporated to dryness under reduced pressure. The material from the Reinecke precipitate obtained at 5° gave a white crystalline solid on treatment with absolute ethanol. Recrystallization from absolute ethanol gave a product of m.p. $218-220^\circ$ and $[\alpha]_D^{20} -76^\circ$ in water. The figures usually quoted for L-proline are m.p. $220-222^\circ$ (Kossel & Dakin, 1904), and $[\alpha]_D^{20} -80^\circ$ (Fischer & Zemplin, 1909) (Found C, 52.3, H, 8.0, N, 12.0. Calc. for proline $\text{C}_5\text{H}_9\text{O}_2\text{N}$ C, 52.2, H, 7.8, N, 12.2 and for hydroxyproline $\text{C}_5\text{H}_9\text{O}_3\text{N}$ C, 45.8, H, 6.9, N, 10.7%). It is concluded therefore that the product was L-proline. About 1 g was obtained from 7 kg of mushrooms.

Oxidation of catechol by tyrosinase in the presence of proline

Fig. 1 shows the course of O_2 uptake when a small amount of catechol was oxidized by excess of tyrosinase under the conditions already specified, in the presence of different molar

proportions of DL proline. No special significance is to be attached to the initial rates of uptake, which were limited by the rate of diffusion of oxygen into the liquid phase, but it is clear that in the presence of proline the uptake of the second atom of oxygen/mol of catechol was greatly accelerated. Provided a sufficient amount of proline was present, the absorption of oxygen was completed within 10 min, and the amount then absorbed corresponds within the limits of experimental error to 2 atoms/mol of catechol. The intense purple end product appeared to undergo no further change during the time of the experiment.

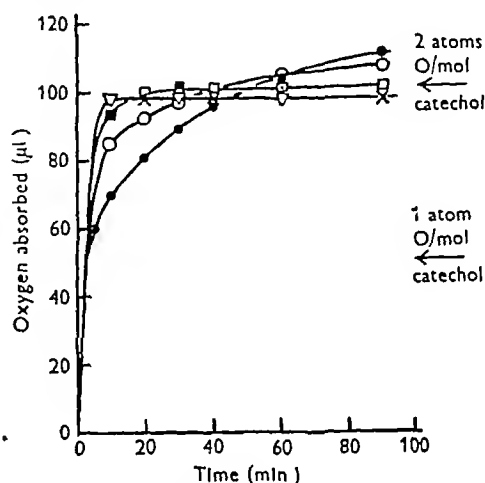


Fig. 1 Oxidation of 0.5 mg catechol by tyrosinase in the presence of proline, at pH 7.0 and 20° . Mol. proline present/mol catechol: ●—●, none; ○—○, 0.5; □—□, 1.0; ▽—▽, 2.0; ×—×, 5.0.

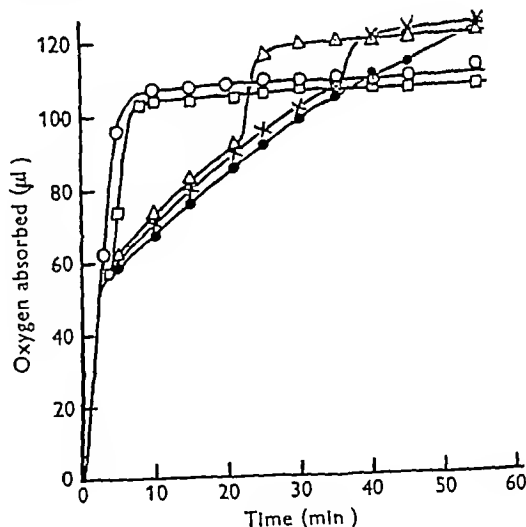


Fig. 2 Oxidation of 2.0 ml 0.0025M catechol by tyrosinase in the presence of 0.025M hydroxyproline at pH 6.0 and 25° . The hydroxyproline was introduced into the reaction mixture: ○—○, before the beginning of the oxidation; □—□, at 4 min; ▽—▽, at 22 min; ×—×, at 36 min; and ●—●, not at all.

When hydroxyproline is substituted for proline in such an experiment the course of O_2 uptake and the colour developed are scarcely distinguishable from those observed in the case of proline. Fig. 2 shows the O_2 uptakes in an experiment in

which the addition of hydroxyproline to the reaction mixtures was delayed for a period after the enzymic oxidation of catechol had begun. Warburg flasks with two side arms were used, the second side arm containing the imino acid solution which was tipped into the main part when the desired time interval had elapsed. The experiment was carried out at pH 6.0 in order to slow down the absorption of the second atom of oxygen. It will be noted that the addition of the imino acid caused in each case a rapid absorption the magnitude of which diminished with increasing delay in imino acid addition, but the total uptake at the end of the experiment was the smaller, and the nearer to precisely 2 atoms/mol catechol, the earlier the imino acid addition. At the end of this experiment each reaction mixture was diluted to 50 ml and its colour intensity measured in the Spekker absorptiometer. The results obtained are shown in Table 1. Little reduction in pigment formation was observed

Table 1 *Effect of delay in hydroxyproline addition on pigment formation in the catechol tyrosinase system*

(Reaction mixture 2.0 ml., pH 6.0, 25° 0.0025M catechol, 0.025M hydroxyproline. Diluted with water to 50 ml for absorptiometer reading.)

Time after beginning of oxidation when hydroxyproline was added (min)	Atoms of O/mol catechol already absorbed at this time	Final colour intensity*
0	0	0.490
4	1.03	0.430
22	1.66	0.279
36	1.95	0.217
No addition	—	0.107

* Absorptiometer reading, filter no. 604

when the addition of hydroxyproline was delayed until 1 atom of oxygen/mol catechol had been absorbed, but the uptake of the second atom of oxygen was accompanied by a corresponding loss of capacity for pigment production upon addition of imino acid. Similar results were recorded by Jackson (1939) in a study of the oxidation of catechol in the presence of aniline, and were interpreted as indicating that only the primary oxidation product of catechol, i.e. o-benzoquinone, reacted with the base to form pigment. The same interpretation fits the facts of the present case.

Oxidation of catechol by tyrosinase in the presence of glycine

The effect of glycine on the oxidation is shown in Fig. 3. In this case, too, the initial very rapid O_2 uptake was greater in the presence of the amino acid. At the lower glycine concentrations the absorption had almost ceased in 50 min., and the total amount absorbed at the end of the experiment was less than in the absence of amino acid, though significantly in excess of 2 atoms/mol catechol. At the highest glycine concentration the rapid phase was followed by a further O_2 uptake which continued at a considerable and almost constant rate until the end of the experiment. The colour developed is an orange pink, and the observations suggest that a fairly stable pigment is formed when catechol and

glycine are in equimolar proportions, and that when glycine is present in excess oxidation of the excess takes place, as indicated by the continued O_2 uptake. This oxidation is accompanied by liberation of NH_3 , as was observed by Platt & Wormald (1927).

A few similar experiments were made with alanine, glutamic acid and arginine, with similar results. In each case, at relatively high amino acid concentrations, the rapid phase of O_2 uptake ending at about 2 atoms/mol catechol was followed by a slower continued absorption indicative of oxidation of the amino acid. The rapidity of this oxidation diminished in the series glycine, arginine, glutamic acid, alanine.

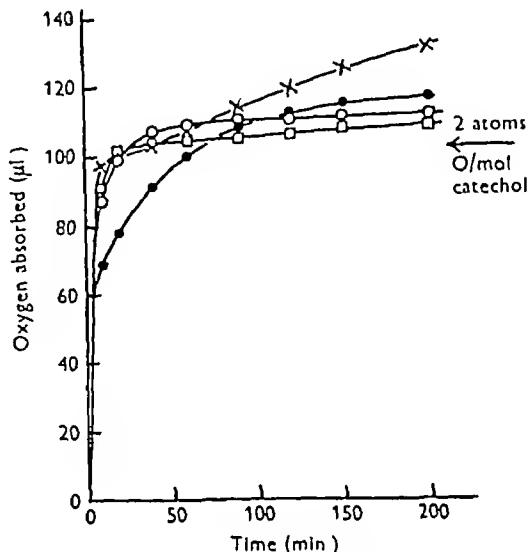


Fig. 3 Oxidation of 0.5 mg catechol by tyrosinase in the presence of glycine, at pH 7.0 and 20°. Mol glycine present/mol catechol: ●—●, none; ○—○, 1.0; □—□, 2.0; ×—×, 5.0.

Oxidation of catechol by tyrosinase in the presence of pyrrolidine

It is of interest to know whether pyrrolidine, like its derivatives proline and hydroxyproline, gives rise to pigment formation and affects the O_2 uptake in the tyrosinase catechol system. Tests showed pyrrolidine to behave like its carboxylated derivatives. The effect of pyrrolidine on the O_2 uptake is seen in Fig. 4, where curves relating to similar experiments with hydroxyproline and with aniline are given for comparison.

Oxidation of homocatechol by tyrosinase in the presence of amino acids

Pigment formation from homocatechol (4-methylcatechol) took place in a manner exactly analogous, so far as could be judged, to its formation from catechol. In Fig. 5 the O_2 uptakes of catechol and homocatechol under the same conditions with and without proline are compared. The reaction rate, particularly as regards the uptake of the second atom of oxygen, was slower in the case of homocatechol, but the amino acid exerted a similar effect with each substrate. When 4.5 dimethylcatechol was present as substrate, the addition of proline had no effect on the colour which developed in the reaction mixture.

Pigment formation from monophenols and imino acids

By virtue of their monophenolase activity tyrosinase preparations are able to form pigment from phenol and *p* cresol. The pigments formed in the presence of the imino

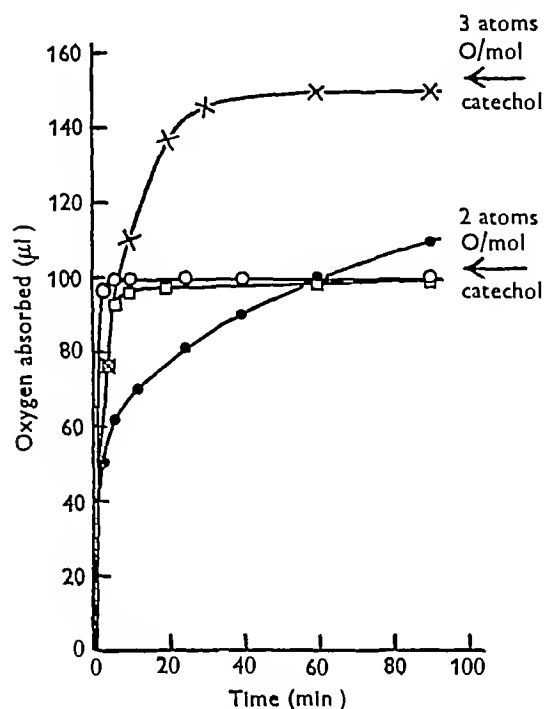


Fig 4 Oxidation of 0.5 mg catechol by tyrosinase at pH 7.0 and 20°C, alone (●-●) and in the presence of excess hydroxyproline (○-○), pyrrolidine (□-□) and aniline (x-x)

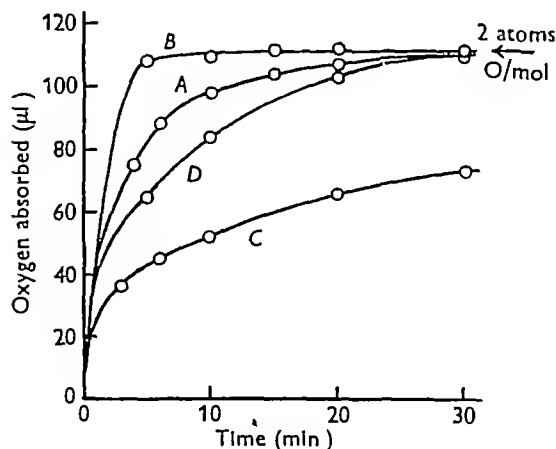


Fig 5 Oxidation of 2.0 ml 0.0025M *o*-diphenols by tyrosinase at pH 7.0 and 25°C, alone and in the presence of 0.025M-proline: A, catechol; B, catechol and proline; C, homocatechol; D, homocatechol and proline

acids appear to be identical with those formed from catechol and homocatechol respectively. The course of O_2 uptake with monophenol as substrate to the enzyme is shown in Fig 6, and is seen to be more rapid in the presence of hydroxyproline

The total uptake is about the expected 3 atoms/mol monophenol. The induction period before the O_2 absorption rate reaches its maximum, a well known characteristic of the oxidation of monophenol by the enzyme, is discernible in the phenol curves, though at the high enzyme concentration used in the experiment it was very brief. It is of interest to note that the duration of this period is much diminished by the presence of the imino acid, an effect which is seen more clearly in experiments with smaller enzyme concentrations.

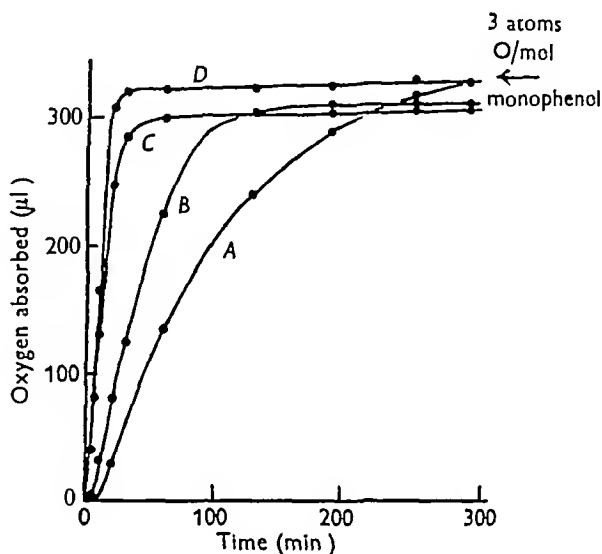


Fig 6 Oxidation of 2.0 ml 0.005M monophenol by tyrosinase at pH 7.0 and 25°C, with and without 0.01M hydroxyproline: A, phenol; B, phenol and hydroxyproline; C, *p*-cresol; D, *p*-cresol and hydroxyproline

In an experiment similar to that to which Fig 6 refers, but including in addition reaction mixtures with equivalent concentrations of catechol and homocatechol instead of monophenol, the pigmented reaction mixtures at the end of the manometric experiment were equally diluted, and an estimate of relative pigment concentrations obtained by taking absorptiometer readings. The readings for the products from phenol and catechol were 0.690 and 0.678 respectively, for those from *p*-cresol and homocatechol 0.546 and 0.460. Thus pigment formation was with the former pair slightly, with the latter pair markedly, more efficient when the substrate was monophenol than when it was the corresponding *o*-diphenol.

Colorimetric study of pigment formation

To obtain information about the relationship between the extent of pigment formation and the molar ratio of imino acid to catechol during the reaction, mixtures containing 5 μ mol catechol and excess enzyme, with varying proportions of imino acid, at pH 7 and 25°C, were shaken with air long enough to ensure maximum colour development, then diluted to 25 ml with water and examined in the Spekker absorptiometer with the Ilford filter no. 604. This procedure was also carried out with homocatechol, and colour development with pyrrolidine, hydroxyproline ethyl ester, glycine, dimethylamine and methylamine, as well as with proline and hydroxyproline, was examined. The results are shown in Figs 7 (a) (catechol) and 7 (b) (homocatechol), where the

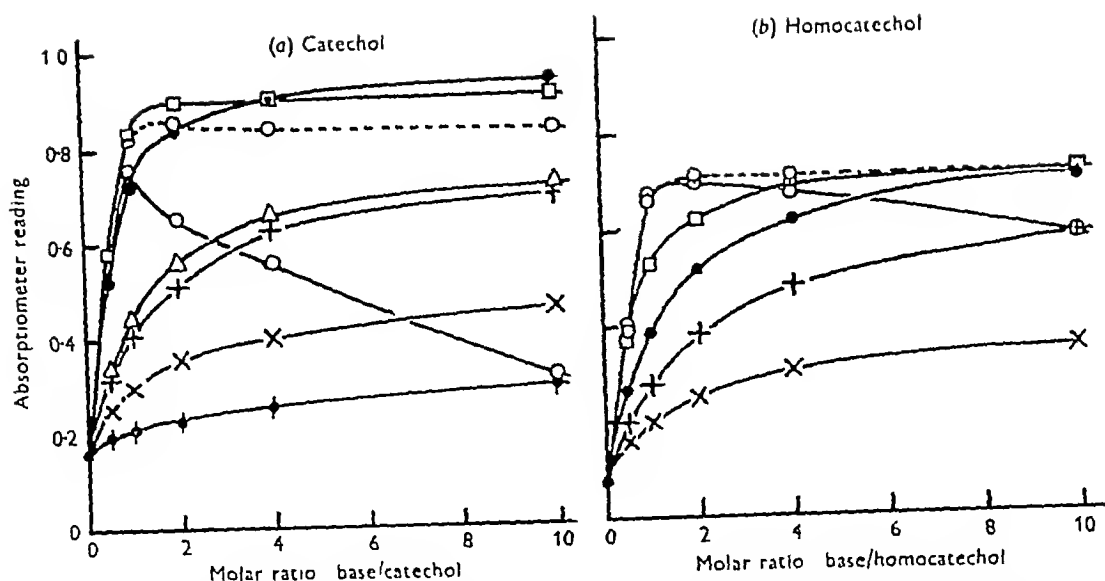


Fig 7 Effect of variation in concentration of nitrogenous base on the colour developed during the oxidation of *o* diphenols by tyrosinase at pH 7.0 and 25° (a), catechol, (b), homocatechol. The broken line represents the results of an experiment in which the pH was 6.0. The bases used were ●—●, proline, □—□, hydroxyproline, ○—○, hydroxyproline ethyl ester, +—+, pyrrolidine, ×—×, glycine, △—△, dimethylamine, †—†, methylamine.

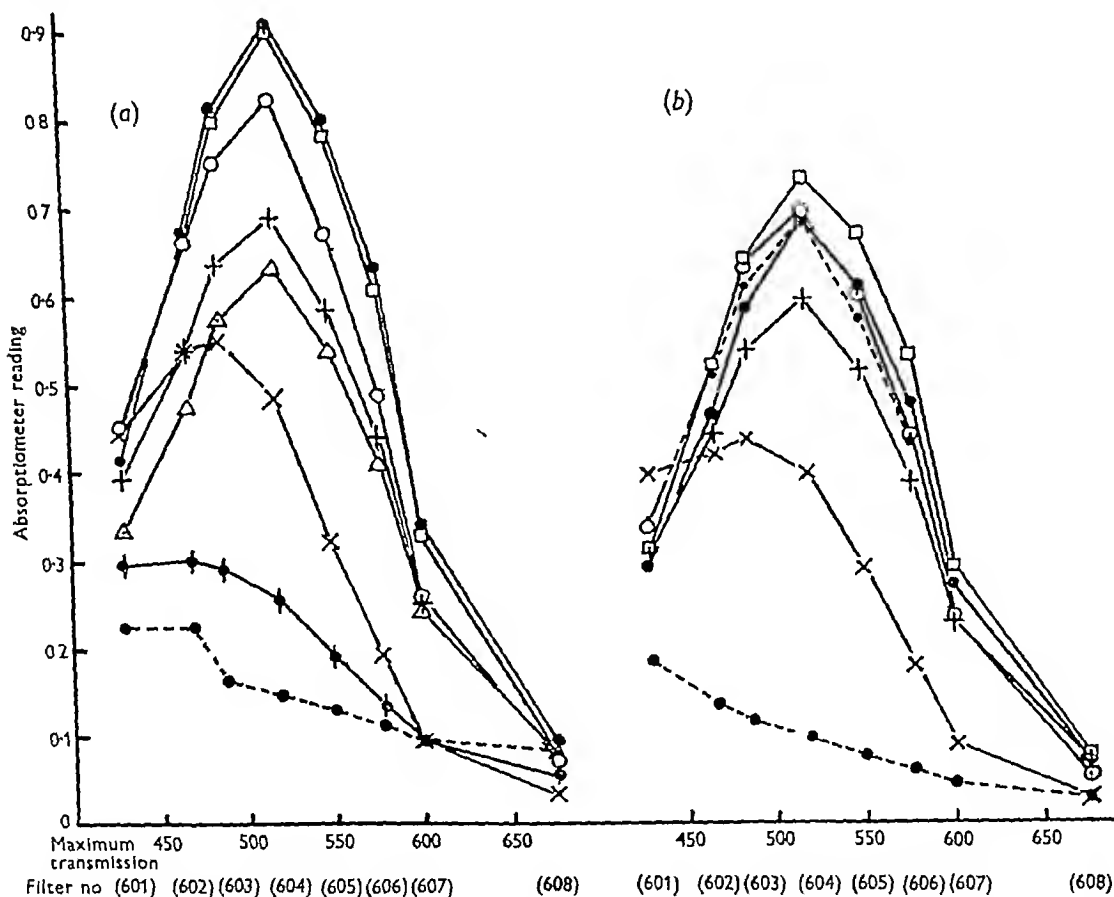


Fig. 8 Light absorption curves of pigments formed by enzymic oxidation of (a) catechol and (b) homocatechol in the presence of nitrogenous bases. Ordinates, absorptometer readings, abscissae, wave lengths of maximum transmission of the filters used, the serial number of each filter (Ilford spectrum series) being given in the appropriate position under the wave length scale. The bases used were ●—●, proline, □—□, hydroxyproline, ○—○, hydroxyproline ethyl ester, +—+, pyrrolidine, ×—×, glycine, △—△, dimethylamine, †—†, methylamine. The broken line at the foot of each set of curves indicates the readings in a control in which no base had been present. The measurements were made at a dilution corresponding to 0.0002M in terms of the amount of *o* diphenol used, and in a 1 cm. cell. The upper broken line in (b) indicates the readings given by a 0.0002M solution of the synthetic quinone I.

colour intensity (in terms of actual absorptiometer reading) is plotted against the ratio mol base/mol *o* diphenol of the reaction mixture. It will be noted that with catechol as substrate increasing the concentration of nitrogenous base above an equimolar proportion results in little increase in pigment formation in the case of proline, hydroxyproline and hydroxyproline ethyl ester, and even with homocatechol more than 50% of the maximum colour is produced by these substances at a 1:1 ratio. This is a strong indication that the formation of the pigment requires only 1 mol of nitrogenous base/mol of *o* diphenol. It seems likely that a condensation reaction takes place between 1 mol of nitrogenous substance, through its imino group, and 1 mol of the primary oxidation product, *o* benzoquinone (Pugh & Raper, 1927), to give the leuco form of the pigment, which is then changed into the coloured form by the utilization of 1 atom of oxygen. In these experiments hydroxyproline ethyl ester behaved uniquely in that increasing its relative concentration above the 1:1 ratio led to a diminution in the amount of pigment found. This effect was much more striking with catechol than with homocatechol as the substrate, and was negligible at pH 6 as the appropriate curve in Fig. 7 indicates. It appears to be due to an instability of the pigment in the presence of excess hydroxyproline ethyl ester. The curves of Fig. 7 do not give such a definite indication as to the molecular proportions involved in the reactions with pyrrolidine, glycine and the methylamines. Higher concentrations of these bases were necessary for effective reaction, and it is uncertain whether an approach to quantitative conversion of *o* diphenol to pigment was achieved even at the highest concentrations used.

The colours given by catechol and homocatechol with the same base were not distinguishable in tint to the naked eye. The various substances containing a secondary amino group appeared to give an identical colour in the reaction, whilst those with a primary amino group gave quite a different hue. To record this more objectively, absorptiometer readings were taken, using in turn each filter of the Ilford spectrum series, and the results are shown in Fig. 8. The pigments derived from secondary amines all give curves of a very similar form with maximum absorption when the green (no. 604) filter was used. The glycine pigment curves are clearly different and show maximal absorption with the blue-green filter (no. 603), and methylamine resembled glycine in its behaviour in this respect. These facts suggest that the pigments derived from the various secondary amines are analogous in structure, but of course do not necessarily preclude a similar structure in the glycine pigment.

Liberation of hydrogen ion during pigment formation

The method used for this study has already been described. The experiments were carried out at room temperature, about 20°. The addition of enzyme to the aerated solution of *o* diphenol and base caused a rapid liberation of hydrogen ion corresponding in time with the rapid phase of O_2 absorption and ceasing abruptly when pigment formation was completed. The course of hydrogen ion liberation in one such experiment is shown in Fig. 9, and the relationship between total hydrogen ion liberated and amount of *o* diphenol oxidized may be seen in the summarized data of Table 2. It is interesting to note that there was a significant liberation of hydrogen ion during the oxidation even in the absence of any nitrogenous base, but the source of this hydrogen ion is at present unknown. The data given in Table 2 are in accordance with the

view that when a base was present the amine group was involved in the pigment forming reaction, and was converted into a less basic form unionized at pH 7, so that an equivalent of hydrogen ion was liberated. Direct quantitative relation of the measured hydrogen ion liberation to the

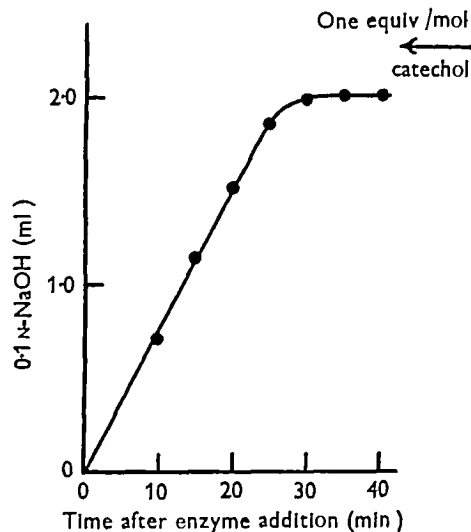


Fig. 9. Hydrogen ion liberation during the oxidation of 0.227 mmol catechol by tyrosinase in the presence of 0.227 mmol hydroxyproline. The reaction took place in unbuffered solution at pH 7.0, and the ordinates represent the addition of standard alkali necessary to maintain the pH constant during the reaction.

Table 2. *Liberation of hydrogen ion during the oxidation of catechol and homocatechol by tyrosinase in the presence of nitrogenous bases*

Molar ratio base/phenol	H ⁺ ions liberated/mol of			
	Catechol		Homocatechol	
	1:1	10:1	1:1	10:1
Nitrogenous base				
Proline	0.76	0.96	0.48	0.81
Hydroxyproline	0.91	1.02	0.72	0.90
Hydroxyproline ethyl ester	0.89	0.95	0.79	0.83
Pyrrolidine	0.55	0.82	0.47	0.73
Dimethylamine	—	0.79	—	—
Glycine	0.52	0.79	—	0.68
Methylamine	—	0.53	—	—
None	0.18		0.23	

amount of amino group reacting depends upon the assumption that the reacting group is fully ionized in its initial, and completely unionized in its final, stage. Of the bases used, only hydroxyproline ethyl ester ($pK = c. 7.5$) has its basic group appreciably unionized at pH 7, and in this case only, therefore, the experiments were carried out at pH 6. In the cases where pigment formation was most efficient, the hydrogen ion liberation approached 1 equiv./mol. of *o* diphenol initially present when the base/*o* diphenol ratio was 10:1, but did not exceed this even when 90% of the maximum was achieved at a 1:1 ratio. It is clear then that only 1 mol of base/mol of *o* diphenol participates in the reaction. The

smaller yields of hydrogen ion in the case of pyrrolidine, glycine and methylamines correspond with the lesser degree of reactivity of these compounds in pigment formation already pointed out in connexion with the colour production curves of Fig 7

Properties of the pigments

Since the preliminary report on the formation of the proline and hydroxyproline pigments was made (Jackson & Kendal, 1940) many attempts at isolation of the pigments have been carried out without success. They are very soluble in water, methanol and ethanol, and are not extracted from aqueous solution into any of the common solvents. They are moderately stable within a narrow range of pH between 6 and 7, but even at pH 7 about 45% of the colour intensity disappears from the solution in 48 hr at room temperature. Outside this range the pigments are very unstable, and this is undoubtedly the main difficulty in attempts at isolation.

The addition of strong acid to a solution of the pigment formed from catechol and imino acid causes rapid and irreversible decolorization, and the kinetics of this decomposition have been studied. Catechol (1 mg) was oxidized under the conditions already described, with excess of enzyme and excess of the imino acid present. The pigment containing reaction mixture (4 ml) was then diluted with 0.1 M-phosphate buffer of pH 7 to a volume of 50 ml. Samples of this solution (5 ml) were mixed with water and varying amounts of 0.1 N-HCl, to give in each case a final volume of 15 ml. Immediately after the addition of the acid the mixture was placed in the absorptiometer cell and readings were taken at timed intervals. When the desired number of observations had been made the pH of the solution was determined with the glass electrode. At pH's of less than 3.0 the pigment was found to be completely decolorized within 10 min. The results are summarized in Fig 10, where the logarithm of the absorptiometer reading is plotted against the time which had elapsed since the pH of the pigment solution was changed from its original value of 7.0 to the value specified alongside each curve. Since the logarithmic plots are straight lines, the conversion of the pigment into colourless products has the appearance of being a monomolecular reaction. However, the relative rates of destruction at different pH's indicate that the disappearance of the colour follows the course of a bimolecular reaction in which $dx/dt = k[H^+](a-x)$, where a is the amount of pigment originally present (as measured by absorptiometer reading) and x is the amount converted to colourless products in time t . The values of k in this expression, calculated from the data of Fig 10, are given in Table 3. The acid decomposition of the pigment formed, when catechol was oxidized in the presence of one of the partially purified fractions of mushroom extract, was examined in the same way. Log k was found to be 2.75, which is in agreement with the belief that the effective substance in the fraction was proline. In similar experiments with the pigments from homocatechol and hydroxyproline or hydroxyproline ethyl ester values for log k of approx 3.1 and 0.4 were respectively obtained. The decolorization of the homocatechol-hydroxyproline pigment, therefore, proceeds at a rate comparable with that of the corresponding catechol pigment, whilst in the case of the ester pigment, the rate is of a different order of magnitude, some hundreds of times slower. This suggests that in the latter case a different mechanism is involved, it may be that the overall rate is determined by the rate of

hydrolysis of the esterified carboxyl group present in this pigment. The pigment formed with pyrrolidine gave quite different results. Acidification of a sufficient degree produced, in this case, an instantaneous change from purple to yellow, and at no pH between 1.0 and 7.0 was any further change in the absorptiometer reading detectable in a period of 10 min. The colour at pH 1.0 was a clear yellow with no trace of pink, and intermediate tints were given as the pH was raised until the full purple colour appeared when a pH of 3.0 was exceeded. The change was reversible and the pigment behaved as a typical indicator. A rough calculation, from the absorptiometer readings, of the proportions of the two forms present at different pH's suggested that the colour change was associated with the ionization of a group with a dissociation exponent of about 2.1, whether the pigment had been prepared from catechol or from homocatechol.

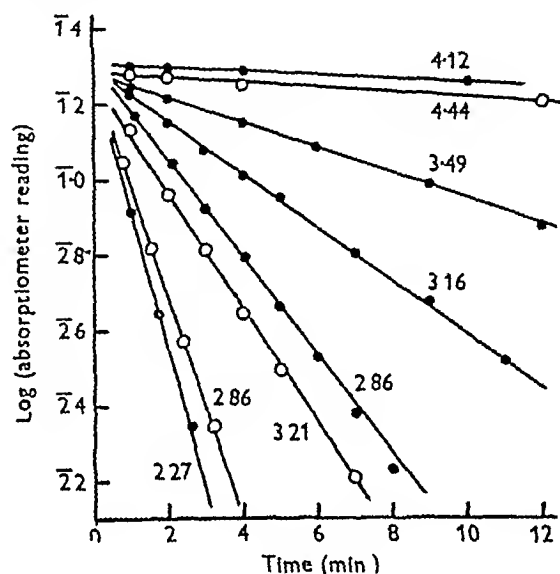


Fig 10 Decolorization of pigments in acid solution. The logarithm of the absorptiometer reading is plotted against the time which had elapsed since the pH of the pigment solution was changed from its initial value of 7.0 to the value specified alongside each curve. The pigments were formed from ●—●, catechol + hydroxyproline, and ○—○, catechol + proline.

Table 3 Velocity constants of the pigment decolorization reaction

Proline pigment		Hydroxyproline pigment	
pH	log k	pH	log k
2.86	2.69	2.27	2.19
3.21	2.76	2.86	2.33
4.44	2.67	3.16	2.36
—	—	3.49	2.39
—	—	4.12	2.18
Mean 2.71		Mean 2.29	

The decomposition of the proline and hydroxyproline pigments in acid solution is accompanied by the evolution of CO_2 . This was demonstrated in experiments in the Warburg apparatus. O_2 uptake during pigment formation was determined in the manner already described. A second manometer with the same reaction mixture had no KOH in its inset cup, and within the vessel was a dangle containing 0.2 ml of

2N HCl, which was tipped into the reaction mixture after the O_2 uptake accompanying pigment formation was complete. From the resultant increase in pressure the volume of CO_2 liberated was calculated in the usual way, due allowance being made for the small initial CO_2 content of the reaction mixture which was determined in blank experiments. A few similar experiments were carried out in which pyrrolidine, glycine or alanine was used instead of the imino acid. In some of these the NH_3 content of the reaction mixture at the end of the acid treatment was determined, the reaction mixture being made alkaline, the NH_3 drawn over in an air stream into standard acid and estimated by back titration. The results of a number of such experiments are given in Table 4. It is evident from the table that the CO_2 liberation on acidification of the imino acid pigments approaches 1 mol/mol of catechol used for pigment formation. Since this is not observed in the case of the pyrrolidine pigment (where the small figure obtained is not with certainty outside the limits of experimental error) it is probable that the CO_2 liberation is the result of instability of the carboxyl group in the pigment in acid solution. In the experiments with the glycine and alanine pigments CO_2 liberation on acidification lagged markedly behind O_2 uptake at the moment of acidification. This may be merely a reflexion of the failure of the approach to quantitative formation of pigment from catechol in the case of these amino acids.

Table 4 *Oxygen uptake during pigment production, CO_2 formation on acidification of pigment solutions, and ammonia content of reaction mixtures after this treatment*

(Pigment was formed from 4.55 μ mol. catechol in each case. In Exps 1-4, 22.7 μ mol. nitrogenous base were present, and the reaction mixture was acidified after O_2 absorption had ceased. In Exps 5-8, 91 μ mol. nitrogenous base were present, and the reaction mixtures were acidified at the times indicated. Zero time was the moment of addition of enzyme. The figures given are mol/mol of catechol.)

Exp no	Nitrogenous base	Time (min)	O_2	CO_2	NH_3
1	Proline	—	0.99	0.92	—
2	Hydroxyproline	—	1.01	0.75	—
3	Mushroom fraction	—	1.01	0.92	—
4	Pyrrolidine	—	1.01	0.09	—
5	Glycine	17	1.10	0.20	0.61
	"	211	3.23	0.97	2.99
6	Glycine	10	0.99	0.15	0.20
	"	70	2.12	0.53	1.85
	"	200	3.23	0.83	3.25
7	Alanine	10	0.88	0.64	0.00
	"	140	1.06	0.77	0.26
	"	220	1.21	0.83	0.51
8	Proline	210	1.01	0.95	0.00

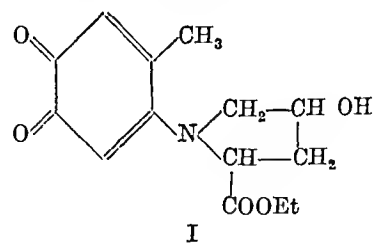
The amounts of NH_3 liberated from the reaction mixtures were in most cases so small as to be within the limits of experimental error of estimation. Only in the later stages of the reaction with glycine can NH_3 liberation be claimed to have been with certainty demonstrated to occur on a scale too great to be accounted for as possibly the result of destruction of the pigment itself by the acid or alkali treatment. It is concluded that this NH_3 was set free in the reaction prior to

the acidification, the amount of it appears to have been about 1 mol/mol of oxygen absorbed. The probable nature of the reactions involved will be referred to in the discussion.

SYNTHESIS OF PIGMENT

In view of the difficulties met with in attempts to isolate the pigments, believed to be due to the instability of the pigments in aqueous solution, some attention was given to the possibility of preparing such pigments by ordinary chemical methods in non-aqueous media. A crystalline pigment was obtained from homocatechol and hydroxyproline ethyl ester in the following way.

Homocatechol (0.31 g) and hydroxyproline ethyl ester hydrochloride (0.45 g) were dissolved in dry ethanol (25 ml) with gentle warming to facilitate solution of the hydrochloride. The addition of dried Na_2SO_4 (3.5 g) followed by dry, alkali-free Ag_2O (1.5 g) resulted in the development of the expected purple-red colour. The mixture was shaken for 10-15 min. Ag_2O , Ag and Na_2SO_4 were removed by filtration and the filtrate cooled in ether solid CO_2 . Scratching promoted the separation of minute rosettes of needles. After cooling (1 hr) the crystals were filtered off and washed with light petroleum (b.p. 40-60°) until the washings were colourless, and then dried *in vacuo*. Recrystallization from absolute ethanol-ether gave 130 mg of purplish-black glistening crystals, m.p. 125-126°. Found C, 60.2, H, 6.3, N, 5.0. $C_{14}H_{17}O_5N$ requires C, 59.8, H, 6.4, N, 5.0%. The product is therefore taken to be 4-(4'-hydroxy-2'-carbethoxy-1'-pyrrolidyl)-5-methyl-o-benzoquinone with the structure I. It will be referred to in this paper as 'the quinone I'.



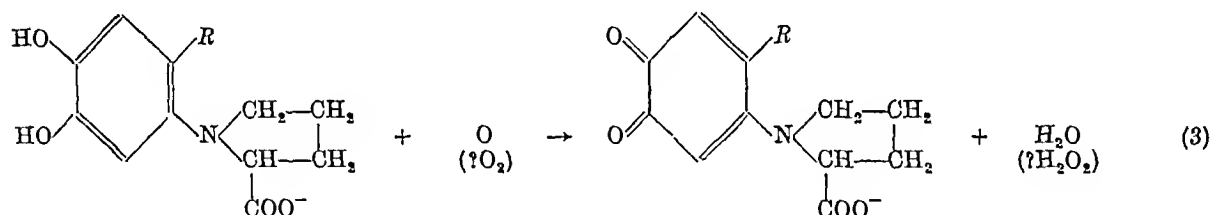
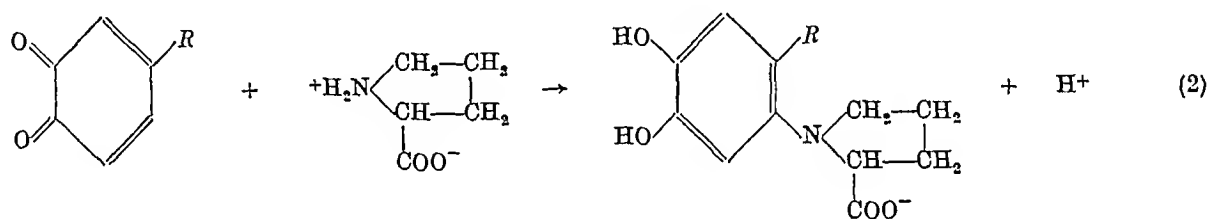
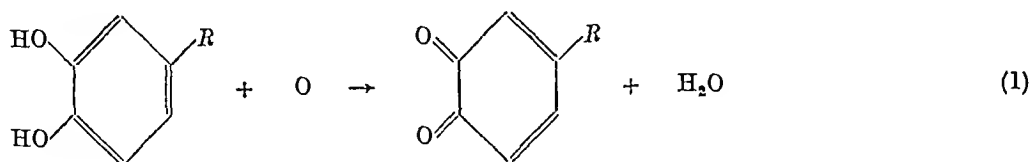
Using catechol instead of homocatechol, or free hydroxyproline or proline instead of the ethyl ester, pigments are readily formed under these conditions, but it has not so far proved possible to isolate them in a pure state. It appears that the presence in the pigment molecule of either the free carboxyl or an unsubstituted position para to the quinonoid oxygen confers upon it an extra degree of instability which renders its isolation more difficult.

Aqueous solutions of the crystalline pigment resemble very closely in colour those of the enzymically produced imino acid pigments, as may be seen on comparing the appropriate curves in Fig. 8. They are all decolorized by ascorbic acid, and in each case the leuco form of the pigment is spontaneously re-oxidized on shaking with air (e.g. after removal of enzyme by adsorption on alumina). A striking property of the imino acid pigments formed in the

enzyme reaction is that they are able to effect a non-enzymic oxidation of glycine by atmospheric oxygen, and this property is also shared by the quinone I. These facts strongly suggest that all these pigments have essentially the same structure

DISCUSSION

The quantitative observations which have been made on oxygen uptake and hydrogen-ion liberation accompanying pigment formation, on the relationship between intensity of colour developed and imino acid concentration, and on CO_2 liberation during acid decomposition of the pigment, suggest that the sequence of reactions involved may be represented as follows



When $R = \text{H}$ (catechol) the sequence is the same as when $R = \text{CH}_3$ (homocatechol), although the speed of the actual pigment-forming reaction (2) is greater. Of these reactions (1) is now generally accepted as the first step in the oxidation of catechol by tyrosinase. In the absence of other substances, the *o*-quinone formed takes part in further reactions which are associated with the uptake of about 1 atom of oxygen per mol. In the belief that this further uptake is precisely 1 atom/mol, Wagreich & Nelson (1938) have suggested that the product is a hydroxy *o*-quinone which then polymerizes to give the brownish-black end product. We have frequently observed a total O_2 absorption of up to 2.3 atoms/mol catechol, and it seems likely that the end stages are more complicated and that they may involve, perhaps in addition to hydroxyquinone formation, some degree of condensation of the type shown by Westerfield & Lowe (1942) to occur during the oxidation of *p*-

cresol by peroxidase. Evidence for the accumulation of *o*-quinone during the early stages of the oxidation of catechol by tyrosinase has been provided by Dawson & Nelson (1938), and the reactions which bring about the disappearance of *o*-quinone are relatively slow compared with primary oxidation of catechol or homocatechol.

In the presence of primary or secondary amines the effectiveness of pigment formation must depend in the first place on the relative speeds of the reaction of *o*-quinone with amine, and the reactions by which the *o*-quinone is removed in the absence of amine. The condensation (reaction 2) under the conditions of our experiments appears to have been sufficiently rapid in the cases of proline, hydroxyproline, and hydroxyproline ethyl ester for pigment formation

from catechol to have exceeded 90%, from homocatechol 80%, of the theoretical requirement according to the reaction scheme proposed. This conclusion is based on the hydrogen-ion liberation during the reactions. The reaction with pyrrolidine, glycine and the methyl amines was much slower and a greater fraction of the *o*-diphenol present was presumably converted into the same end products as are obtained in the absence of nitrogenous base.

It is well established that when catechol is oxidized by tyrosinase in the presence of aniline, dianilinoquinone is formed almost quantitatively (Pugh & Raper, 1927). It is not clear why in the case of the imino acids, reaction (2), analogous to the first stage of dianilinoquinone formation, having taken place, the quinone derived from the product by reaction (3) does not then condense with a second molecule of imino acid. No study of the products of the reaction between quinone and free aliphatic

amino-acids appears to have been made Fischer & Schrader (1910), allowing glycine ethyl ester to react with *p*-benzoquinone in ethanol, isolated the product and showed it to be the diethyl ester of diglycinoquinone. These workers prepared in the same way the ester of dialaninoquinone, and from *p*-toluquinone the ester of diglycinotoluquinone. The formation of the diglycino compound from toluquinone in this case is interesting, particularly in view of Suchanek's (1914) finding that whereas *p*-benzoquinone with *p* aminobenzoic acid gave a mixture of mono- and di anilinoquinones, from toluquinone under the same conditions only the monoanilinoquinone was formed, while xyloquinone gave no anilinoquinone. Studies of this kind do not seem to have been made with *o*-benzoquinone, but from a reaction mixture in which *p*-cresol had been oxidized by tyrosinase in the presence of aniline Pugh & Raper (1927) isolated a dianilinoquinone-anil. The conditions under which condensations of the anilinoquinone and quinone-anil types take place do not yet appear to be very well defined. But as regards the former type of condensation the available evidence suggests that it takes place with a strong preference in a position para to a quinonoid oxygen in an *o* quinone, and ortho to a quinonoid oxygen in *p* quinone, without ruling out the possibility of a further condensation in a position not so situated. In the formation of the crystalline pigment prepared synthetically from homocatechol and hydroxyproline ethyl ester in the present series of experiments, the condensation was undoubtedly limited to 1 mol of base per mol homocatechol. There is no reason to question the identity of this pigment with the pigment formed from the same precursors enzymically and both resemble so closely the pigments formed from catechol and the secondary amines in both properties and manner of formation, that it is certain that they all have the same general structure. In the case where catechol is the substrate, it seems possible that steric hindrance by the proline residue may be responsible for the prevention of the condensation of a second molecule of imino acid in the unoccupied position para to quinonoid oxygen. The reaction of *o*-quinone with glycine appears to take place less rapidly than the corresponding reaction with imino acid, and the observations on pigment formation in the catechol-tyrosinase-glycine system do not indicate clearly the molar ratio of catechol and glycine reacting to form the pigment. It is likely that the chief reaction of glycine with *o*-quinone in aqueous solution is the same as in the case of the imino acids so that a monoglycinoquinone is formed.

A suggestion that the enzyme tyrosinase was able to bring about the oxidative deamination of amino-acids was put forward by Chodat & Schweizer (1913) and received some support from other workers

Happold & Raper (1925), however, made it clear that well dialyzed tyrosinase preparations had no effect on amino acids other than tyrosine itself, but demonstrated liberation of ammonia from glycine and alanine by tyrosinase plus *p*-cresol, catechol or phenol. They attributed the oxidation of amino acid to a secondary reaction of this with *o* quinone formed by the enzyme, and showed that *o* quinone reacted non enzymically with amino acids and liberated ammonia. The fact that the reaction mixtures were 'deeply pigmented' was mentioned, but without comment. The observations recorded in this paper now indicate that *o* quinone condenses with 1 mol of amino- or imino acid to give a leuco pigment which is readily autooxidizable in air. Whether water or hydrogen peroxide is the other end product of this autooxidation has not yet been determined. In the case of the imino acid pigments no further reaction takes place even if an excess of the imino acid is present. When the pigment is formed from glycine, and to a lesser extent when it is formed from other amino acids, excess amino acid is oxidized with liberation of ammonia. The relation between oxygen used and ammonia liberated in experiments of this kind with glycine (Table 4) are such as would be expected if the main end products of the oxidation of glycine were ammonia and oxalic acid. Experiments now in progress, which will be described in detail in another paper, indicate that the imino acid pigment reacts with and brings about the oxidation of glycine, and that no enzyme is required for this reaction. It therefore appears probable that the effective oxidant of excess amino-acid is a pigment with a structure corresponding to that of the synthetic quinone I. This may react with further amino acid, dehydrogenating it to give imino acid which then decomposes with liberation of ammonia. The leuco pigment simultaneously formed is spontaneously re oxidized by oxygen. But the possibility that an intermediate stage in the dehydrogenation is a condensation of a second molecule of amino-acid into the pigment molecule should be borne in mind.

An interesting problem arises in connexion with the decomposition of the proline and hydroxyproline pigments in acid solution. This decomposition has been found to be irreversible and to be accompanied by the loss of 1 mol of carbon dioxide. Simple decarboxylation of the proline pigment should, however, yield the corresponding pyrrolidine pigment. However, the latter when prepared in solution directly from catechol and pyrrolidine was found to be reasonably stable, though yellow in colour, in acid solution, giving the purple form reversibly on raising the pH, and hence was clearly not identical with the decomposition product of the proline pigment. It is not at present possible to offer an adequate solution of this problem.

SUMMARY

1 The component of crude mushroom extracts which is responsible for the appearance of a purple colour when such extracts oxidize catechol has been identified by isolation of L-proline

2 The pigment-forming reaction has been studied in respect of colour intensity developed, oxygen absorbed and hydrogen ion liberated, in systems containing purified tyrosinase, catechol or homo catechol, and one of the following proline, hydroxyproline, hydroxyproline ethyl ester, pyrrolidine, glycine, dimethylamine and methylamine. A few experiments were also carried out with alanine, glutamic acid and arginine. The same intense purple colour was obtained in each case in which the nitrogenous substance present had a secondary amino group, the compounds with primary amino

nitrogen gave a much less intense orange-red colour

3 From homogatechol and hydroxyproline ethyl ester, by oxidation with silver oxide in ethanol, 4-(4'-hydroxy-2'-carbethoxy-1'-pyrrolidyl) 5 methyl-o-benzoquinone has been prepared in a pure state. It appears to be identical with the pigment formed from the same precursors in the enzymic reaction mixture, and it is concluded that all the pigments have an analogous structure, and that the pigment-forming reaction is essentially a condensation between 1 mol of o-quinone and 1 mol of nitrogenous base to give a leuco pigment which is spontaneously oxidized to the coloured form by oxygen

4 In the case of glycine, when this is present in excess, the formation of pigment is followed by oxidation of the excess amino acid, with liberation of ammonia. Further oxidation of this type does not take place in the case of the imino acid pigments

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β -Glucuronidase as an Index of Growth in the Uterus and other Organs

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The β -glucuronidase activity of mouse liver or kidney has been shown to be related to the degree of cell proliferation in progress (Levy, Kerr & Campbell, 1948). It was suggested that the rise in uterine glucuronidase observed after administration of oestrogens to ovariectomized mice (Fishman & Fishman, 1944, Fishman, 1947) could also be explained by cell proliferation.

A comparative study has been made of the kinetics of hydrolysis of phenylglucuronide by β -glucuronidase from mouse uterus, liver and kidney, and of the effects on the enzyme activities of various measures designed to produce proliferative changes in one or more of these organs. Mills (1947) showed that ox spleen glucuronidase could be

separated into two fractions, A and B, with slightly different pH optima for the hydrolysis of methylglucuronide. Both these fractions have been found in mouse liver and kidney, while uterine glucuronidase appears to be composed entirely of A. No evidence has, however, been obtained to suggest that the effect of an extrinsic agent on the glucuronidase activity of an organ is dependent upon which fraction happens to be present. As in liver and kidney, changes in the enzyme level in uterus resulting from a variety of causes appear to be associated with alterations in growth.

In the course of these experiments, some unexpected changes in glucuronidase activity were encountered. In ovariectomized mice, measures

designed to cause a rise in glucuronidase in liver also produced an increase in uterus, whilst an elevated enzyme activity in liver as well as in uterus was seen after administration of oestrone. These effects have been further investigated.

A preliminary account of part of this work has been published elsewhere (Kerr & Levy, 1948).

EXPERIMENTAL

Enzyme assay To permit determination of β glucuronidase activity in a single mouse uterus, the procedure previously described (Kerr, Graham & Levy, 1948) was adapted for use with the microcells of the Spekker absorptiometer. As before, the tissue homogenate was freed from inactive protein by maintaining it at pH 5.2 and 38° for 30 min, and the enzyme was precipitated by addition of an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. The enzyme was dissolved in a volume of water such that 0.2 ml of the resulting solution gave a final reading of 2–4 μg phenol after correction for blanks. This volume of the enzyme solution was added to 0.1 ml 0.06M-phenylglucuronide and 0.1 ml 0.1M citrate buffer at the appropriate pH (see below). After incubation of the hydrolysis mixture for 1 hr at 38°, 0.5 ml of a 1 in 5 dilution of Fohn Ciocalteu reagent was added. Protein was removed by centrifuging, and 0.5 ml of supernatant transferred to a tube containing 0.5 ml 1.33N Na_2CO_3 . Colour development was carried out for 20 min at 38°, and the results were read from a graph constructed with standard phenol solutions put through the same procedure. Assays were done in duplicate, and enzyme and substrate controls were performed as usual. This technique was also adopted for determinations of liver and kidney glucuronidase in the experiments described below, and results are shown in terms of glucuronidase units (G.U.)/g moist tissue, where 1 G.U. liberates 1 μg phenol under the standard conditions.

Weight of uterus Before determining the moist weight of uterus, the tissue was freed from intrauterine fluid by pressing it between pieces of filter paper. The figure then obtained was found to bear a constant relation to the weight after drying at 110° for all conditions of the uterus. No error was introduced into the enzyme assay since the intrauterine fluid contained no detectable amounts of glucuronidase.

RESULTS

Kinetic studies The pH-activity curve for hydrolysis of phenylglucuronide by mouse-kidney glucuronidase resembled those previously obtained for liver and spleen (Kerr *et al.* 1948) in having two peaks, one at pH 4.5 and the other at pH 5.2. In the case of uterus, however, the activity curve was symmetrical about pH 4.5, and this was still true when the initial purification of the homogenate was omitted. Changes in the enzyme activity in liver, kidney or uterus were not associated with any alteration in the shape of the pH-activity curve (Figs 1–3). To cover the pH range it was necessary in the case of uterus to pool preparations from two or more mice. For liver and kidney this was only necessary with infant mice. The high figure for

uterine glucuronidase in infant mice supports the view that in this organ, as in others, the activity of the enzyme is a measure of growth processes.

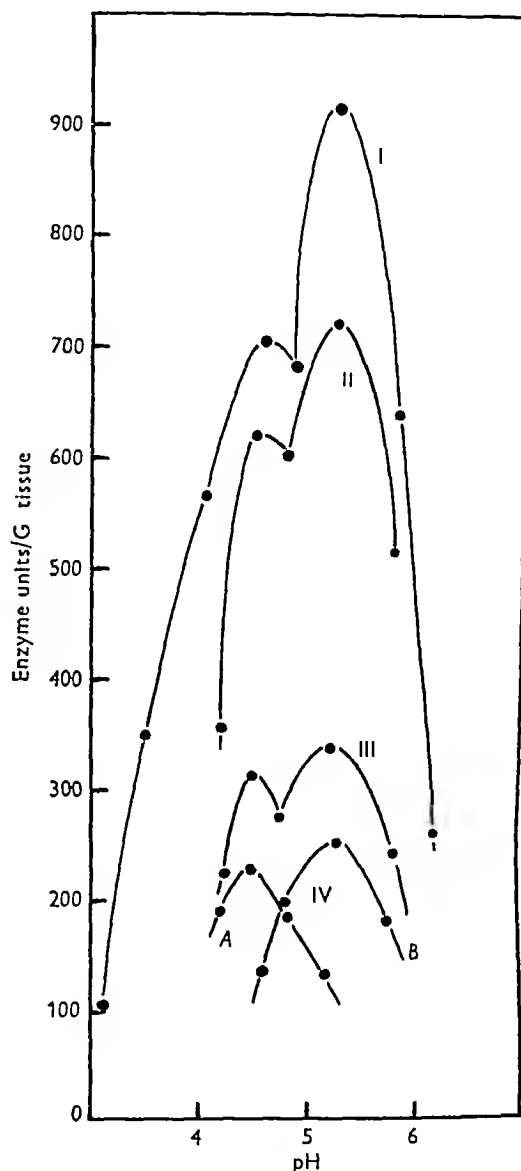


Fig 1 pH-Activity curves for liver glucuronidase I, 6 day old mice, II, adult, 1 day after subcutaneous injection of 5 g CCl_4/kg , III, normal adult, IV, the same preparation as III after separation of fractions A and B.

It was considered that the shapes of the curves for the hydrolysis of phenylglucuronide by mouse liver or kidney indicated the presence of the two glucuronidase fractions found by Mills (1947) in ox spleen, and his technique was applied to their separation. After the preliminary removal of inactive protein by incubation for 30 min at pH 5.2, the homogenate was made 31.5% saturated with ammonium sulphate. The precipitate thus obtained was devoid of glucuronidase activity. On bringing the preparation to 38.5% saturation with ammonium sulphate, a

large part of the enzyme was precipitated (fraction A), whilst all residual activity was removed from solution when the ammonium sulphate concentration was increased to 44.0% saturation (fraction B). The separation of the two peaks in the pH-activity curves for liver and kidney achieved in this way is illustrated in Figs 1 and 2, and it appears that the shapes of the original curves can in fact be explained in terms of Mills's (1947) two fractions. In the fractionation of uterine preparations, all enzyme activity was found in fraction A.

pH activity curves for mouse liver, kidney and spleen

The effects of various agents on fractions A and B in liver and kidney

The possibility was considered that differences between glucuronidase fractions A and B in their distribution and response to extrinsic agents might explain the selective actions of such agents on various organs. The nature of the effect of carbon

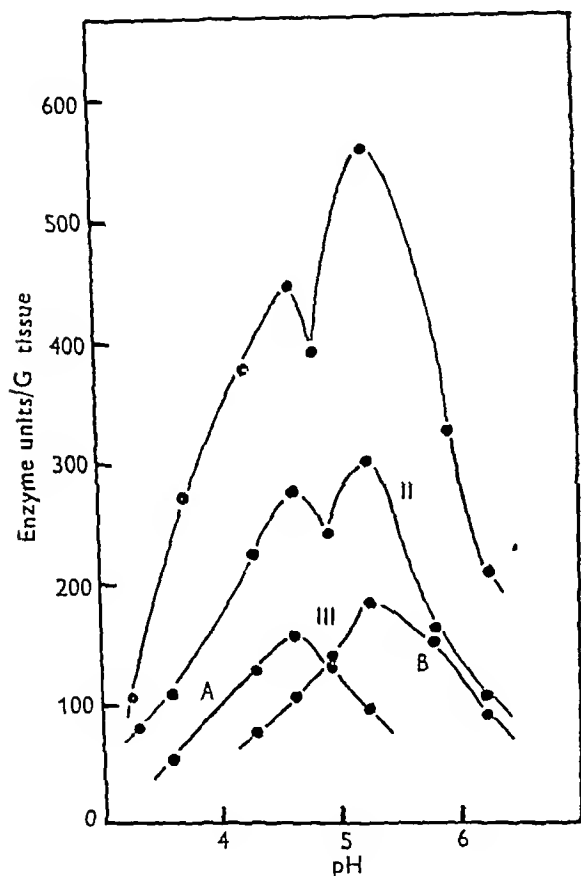


Fig 2 pH Activity curves for kidney glucuronidase I, 6 day old mice, II, normal adult, III, the same preparation as II after separation of fractions A and B

The effect of varying the substrate concentration was studied with uterine enzyme and with fractions A and B from liver. In every case the activity curve closely resembled that obtained with liver before separation of the two fractions (Kerr *et al* 1948). K_m , the substrate concentration at which half the observed maximum velocity of hydrolysis was attained, was approximately the same for the two glucuronidase fractions at the figure for the total enzyme in liver (0.0035M).

Mills (1948) has recently published figures for the pH optima in the hydrolysis of phenylglucuronide by his two glucuronidase fractions from ox spleen, and these correspond exactly with the peaks in the

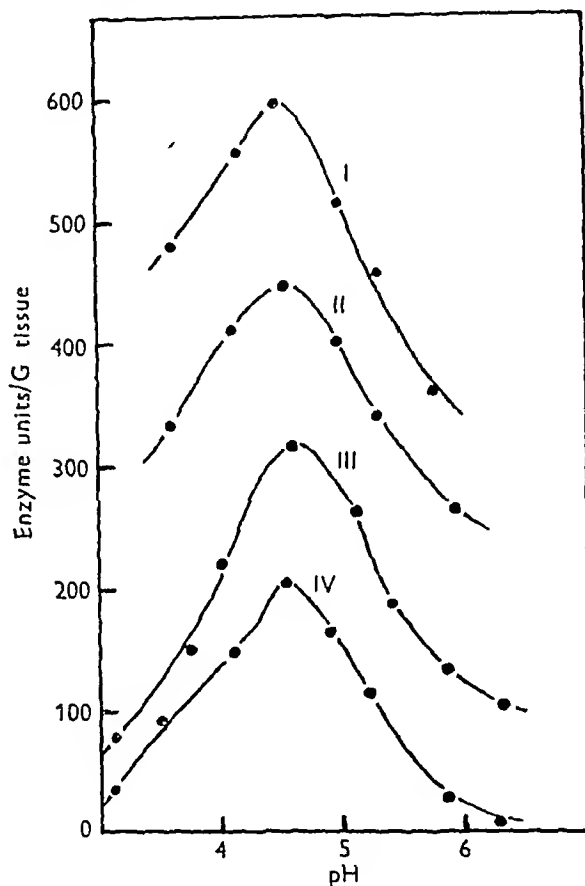


Fig 3 pH Activity curves for uterine glucuronidase I, 10 day old mice, II, ovariectomized adults, 3 days after subcutaneous injection of 17 mg oestrone/kg, III, normal adults, IV, ovariectomized adults

tetrachloride on the pH-activity curve for liver (Fig 1) renders this possibility unlikely, since both fractions were equally affected in the increase in activity. Carbon tetrachloride is known to be without effect on kidney glucuronidase (Levy *et al* 1948), although this organ resembles liver in the composition of the enzyme. In spite of these findings a great many more experiments were done before the possibility just outlined was rejected. In these experiments, the homogenate from each organ was divided into two portions, in one of which A and B were separated as described above and determined at their respective pH optima, 4.5 and 5.2. The other

portion of the homogenate was brought to 50% saturation with ammonium sulphate and the total enzyme thus precipitated was determined at pH 5.2. Average results for liver and kidney under a variety of conditions are shown in Table 1. Since *A* was determined at a different pH from the total activity, figures for the latter do not agree with the sums of the two fractions. One point not brought out in the table is that individual mice, treated and untreated, showed considerable variation in both liver and kidney in the ratio of the two fractions. Very occasionally, one animal in a group displayed complete lack of *A* or *B* in one of the two organs examined, unaccompanied by any compensatory increase in the activity of the remaining fraction. When this occurred, the size of the group was reduced by one in calculating the average and standard error for the fraction in question, as shown in Table 1.

cutaneous injection of carbon tetrachloride in olive oil were in entire agreement with the conclusions arrived at above, in that this agent showed no discrimination between the two fractions in liver, and was without effect on either in kidney. After subcutaneous injection of mercuric nitrate as an aqueous solution, *A* and *B* rose and fell together in kidney as repair processes became active and were completed. Since mercuric nitrate has little effect on liver (see Table 3), its action on this organ was not studied in the present experiments.

Changes in liver glucuronidase after subcutaneous injection of chloroform in olive oil closely resembled, as one might expect from previous work, those produced by carbon tetrachloride. Taking the results as a whole, there was a suggestion that fraction *A* returned to normal more rapidly than *B*. Before dealing with the effects of chloroform on kidney

Table 1 *Effects of various agents on glucuronidase fractions A and B in liver and kidney*

(All values are given as mean \pm S.E., followed (in parentheses) by the number of animals in the group.)

Agent	Sex*	Days after treat ment	G U / g moist tissue								
			Liver			Kidney					
			A†	B†		Total‡	A†	B†		Total‡	
None	M	—	106 ± 17 (6)	223 ± 12 (6)	281 ± 20 (6)	124 ± 16 (6)	123 ± 38 (6)	266 ± 31 (6)			
	F	—	115 ± 12 (6)	247 ± 38 (6)	334 ± 48 (6)	109 ± 8 (6)	118 ± 16 (6)	266 ± 39 (6)			
	cM	—	127 ± 13 (6)	217 ± 18 (6)	301 ± 18 (6)	130 ± 22 (6)	194 ± 18 (6)	286 ± 20 (6)			
	cF	—	116 ± 12 (6)	167 ± 47 (6)	250 ± 49 (6)	113 ± 10 (6)	130 ± 22 (6)	261 ± 43 (6)			
Carbon tetra chloride (5.3 g / kg)	M	1	545 ± 18 (3)	654 ± 40 (2)§	830 ± 62 (3)	122 ± 27 (3)	150 ± 61 (3)	206 ± 28 (3)			
	M	4	364 ± 58 (3)	537 ± 27 (3)	763 ± 65 (3)	118 ± 19 (3)	138 ± 18 (3)	257 ± 19 (3)			
	cF	4	499 ± 109 (3)	588 ± 24 (3)	664 ± 57 (3)	126 ± 14 (3)	158 ± 6 (3)	235 ± 23 (3)			
	cF	7	517 ± 27 (3)	568 ± 35 (3)	693 ± 31 (3)	127 ± 14 (3)	186 ± 70 (3)	270 ± 42 (3)			
Mercuric nitrate (20 mg / kg)	M	3	—	—	—	269 ± 35 (3)	319 ± 55 (3)	463 ± 59 (3)			
	M	6	—	—	—	122 ± 14 (3)	158 ± 15 (2)§	277 ± 19 (3)			
	F	3	—	—	—	224 ± 24 (3)	351 ± 45 (3)	491 ± 62 (3)			
	F	6	—	—	—	122 ± 10 (3)	124 ± 26 (3)	237 ± 61 (3)			
Chloroform (2 g / kg)	M	1	343 ± 188 (6)	766 ± 147 (6)	881 ± 163 (6)	134 ± 19 (6)	121 ± 17 (6)	206 ± 19 (6)			
	M	4	260 ± 42 (3)	561 ± 44 (3)	766 ± 46 (3)	128 ± 18 (3)	234 ± 22 (3)	302 ± 12 (3)			
	M	7	184 ± 29 (6)	559 ± 38 (6)	650 ± 72 (6)	132 ± 29 (6)	395 ± 54 (6)	524 ± 38 (6)			
	F	1	537 ± 64 (6)	410 ± 194 (6)	812 ± 91 (6)	173 ± 10 (6)	135 ± 9 (6)	227 ± 23 (6)			
	F	4	271 ± 45 (3)	570 ± 52 (3)	696 ± 61 (3)	126 ± 19 (3)	148 ± 35 (3)	238 ± 27 (3)			
	F	7	151 ± 42 (6)	559 ± 106 (6)	705 ± 64 (6)	108 ± 16 (6)	223 ± 78 (6)	310 ± 55 (6)			
	cM	1	453 ± 39 (3)	537 ± 25 (3)	659 ± 174 (3)	119 ± 12 (3)	141 ± 17 (3)	221 ± 38 (3)			
	cM	7	152 ± 20 (3)	549 ± 42 (3)	673 ± 32 (3)	132 ± 20 (3)	124 ± 22 (3)	238 ± 30 (3)			
	cF	1	277 ± 22 (2)§	594 ± 32 (2)§	515 ± 23 (3)	187 ± 21 (6)	256 ± 29 (6)	398 ± 67 (6)			
	cF	4	224 ± 25 (3)	510 ± 20 (3)	620 ± 26 (3)	189 ± 15 (3)	247 ± 18 (3)	352 ± 24 (3)			
	cF	7	359 ± 130 (5)	569 ± 39 (5)	657 ± 78 (5)	175 ± 32 (7)	260 ± 69 (7)	359 ± 67 (7)			

* c = castrated

† One G U (glucuronidase unit) liberates 1 μ g phenol from 0.015 M phenylglucuronide in 1 hr at 38° and pH 4.5

‡ One G U (glucuronidase unit) liberates 1 μ g phenol from 0.015 M phenylglucuronide in 1 hr at 38° and pH 5.2

§ One animal in group devoid of this fraction

From Table 1, it appears that in either liver or kidney the distribution of glucuronidase activity between the two fractions was on the average the same for normal male and female mice. Castration 3–4 weeks before sacrifice had no marked effect on the results in either sex.

Results obtained during the prolonged rise in liver glucuronidase activity which follows sub-

cutaneous injection of carbon tetrachloride in olive oil were in entire agreement with the conclusions arrived at above, in that this agent showed no discrimination between the two fractions in liver, and was without effect on either in kidney. After subcutaneous injection of mercuric nitrate as an aqueous solution, *A* and *B* rose and fell together in kidney as repair processes became active and were completed. Since mercuric nitrate has little effect on liver (see Table 3), its action on this organ was not studied in the present experiments.

Changes in liver glucuronidase after subcutaneous injection of chloroform in olive oil closely resembled, as one might expect from previous work, those produced by carbon tetrachloride. Taking the results as a whole, there was a suggestion that fraction *A* returned to normal more rapidly than *B*. Before dealing with the effects of chloroform on kidney

differences in the structure of Bowman's capsule described by Crabtree (1941), but chloroform necrosis, which involved the convoluted tubules, did not extend to the capsule itself. In previous work on glucuronidase (Levy *et al.* 1948), the effect of chloroform on the kidney-enzyme level in normal mice was seen to be confined to males. From the figures for total kidney glucuronidase in Table 1, it appears that the response in the male was abolished by castration 3 weeks previously, although this operation was not performed until the animals were adult. There was no necrosis, and Bowman's capsule had become predominantly female in character. In ovariectomized females, chloroform caused a small, but significant rise in the kidney enzyme, associated with necrosis and repair (for results one day after injection, $P=0.02$, grouping results for all three time intervals, $P<0.01$). Extending the period between ovariectomy and injection of chloroform from 3 to 13 weeks did not appreciably affect this response, but in the interval a change towards the male type of kidney became much more pronounced. As already noted, figures for uninjected mice showed no variations in *A* or *B*, corresponding to the changes in kidney morphology. The fact that in the male kidney the rise in glucuronidase appeared to be confined to fraction *B* may reflect an uneven distribution of the two fractions throughout this organ, with predominance of *B* in the convoluted tubules. In a histochemical study, Friedenwald & Becker (1948) found greater glucuronidase activity in rat-kidney tubules than in the glomeruli, when hydrolysis of suitable glucuronides in unfixed, frozen sections was allowed to proceed at pH 5.

The effects of sex hormones on liver and uterine glucuronidase

Fishman (1947) has examined the effects of testosterone propionate and oestradiol benzoate, separately and in combination, on uterine glucuronidase in ovariectomized mice. In the doses used, testosterone did not antagonize the action of the oestrogen in causing a rise in the enzyme level, and Fishman interpreted this as indicating 'a unique type of specificity of action by the oestrogen'. His results, however, show that administration of the androgen along with the oestrogen did not entirely prevent an increase in the wet weight of the uterus. By itself, testosterone produced a rise in glucuronidase activity and an increase in weight. Fishman's results seem entirely compatible with the view that an increase in glucuronidase activity in uterus, as elsewhere, reflects increased growth, and that his failure to observe antagonism between oestrogen and androgen resulted from use of too great an excess of the latter. Figures for uterus shown in Table 2 bear out this argument.

Oestrone and testosterone were given as single subcutaneous injections of the solutions in olive oil, alone or within 3 hr of each other. Four days after injection of ovariectomized mice with 1.7 mg

oestrone/kg, uterine glucuronidase activity and weight were much greater than in untreated controls. The effects of 0.3 mg oestrone/kg and 3.3 mg testosterone/kg were alike in that there was a comparatively small rise in uterine weight, with a barely perceptible increase in the enzyme activity ($P=0.1$ and 0.02 respectively at the maximum activity). In a smaller dose (2 mg/kg), testosterone had no action on the uterus, but this dose completely antagonized the effects of the larger dose of oestrone on the enzyme and the weight.

In the experiments with oestrone and testosterone, glucuronidase was determined in liver and kidney as well as in uterus. In ovariectomized mice a marked rise in liver glucuronidase, preceding that in uterus, was observed after injection of oestrone in a dose of 1.7 mg/kg (Table 2). This effect was also seen in normal and castrate males, but was absent in intact females, even after 4.3 mg oestrone/kg. Reducing the dose of oestrone to 0.3 mg/kg abolished the action on liver in ovariectomized mice. Histological examination revealed intense mitotic activity, with little evidence of damage in the livers of oestrone-treated castrate males and females. In normal males, the effect on mitosis was slight, but there was a marked increase in binucleate cells. Testosterone, itself without any action on the liver, antagonized the stimulant effect of oestrone on the enzyme and on cell division. Bullough (1946) has studied the effects of oestrone on mitotic activity throughout the body of the adult female mouse, and concluded 'that those substances which have come to be called oestrogenic or female sex hormones are in fact general mitosis stimulators'. Oestrone produced no effect on liver in his experiments, which were, however, confined to the normal female.

Fractionation of the glucuronidase preparations was carried out in many of the experiments listed in Table 2. Fractions *A* and *B* were both involved in the liver response to oestrone, while all uterine activity was invariably found in fraction *A*.

The effects of liver regeneration on uterine enzyme and weight

Administration of carbon tetrachloride to rats has been shown to produce an increase in the weight of the uterus in immature animals (Talbot, 1939), and to enhance the effectiveness of administered oestrone in ovariectomized animals (Pincus & Martin, 1940). Partial hepatectomy causes a similar increase in the potency of administered oestrogen (Segaloff, 1946). From the work of Roberts & Szego (1947) it appears that increased sensitivity to oestrogens occurs during active liver regeneration rather than in the initial stages of injury. In all these studies the animals were treated with an oestrogen or alternatively the ovaries were still present.

It was, therefore, with surprise that increases in the β -glucuronidase activity and the weight of

Table 2 *Effects of oestrone and testosterone on liver, kidney and uterine glucuronidase*(All values are given as mean \pm s.e., followed (in parentheses) by the number of animals in the group)

Treatment	Sex*	Days after treatment	Total α v /g tissue			Uterine weight (mg)
			Liver†	Kidney‡	Uterus†	
None	F	—	334 \pm 48 (6)	266 \pm 39 (6)	333 \pm 53 (6)	234 \pm 56 (6)
	cF	—	250 \pm 49 (6)	261 \pm 43 (6)	174 \pm 45 (6)	34 \pm 18 (6)
	M	—	281 \pm 20 (6)	266 \pm 31 (6)	—	—
	cM	—	301 \pm 18 (6)	286 \pm 20 (6)	—	—
Oestrone (4.3 mg/kg)	F	1	258 \pm 39 (3)	370 \pm 33 (3)	463 \pm 41 (3)	167 \pm 66 (3)
	F	4	244 \pm 32 (3)	357 \pm 44 (3)	439 \pm 63 (3)	192 \pm 76 (3)
Oestrone (1.7 mg/kg)	F	1	365 \pm 60 (3)	223 \pm 47 (3)	388 \pm 45 (3)	320 \pm 24 (3)
	F	4	267 \pm 26 (3)	253 \pm 41 (3)	441 \pm 87 (3)	253 \pm 31 (3)
	cF	1	431 \pm 42 (3)	313 \pm 17 (3)	300 \pm 54 (3)	26 \pm 5 (3)
	cF	2	481 \pm 34 (3)	275 \pm 67 (3)	343 \pm 47 (3)	47 \pm 8 (3)
	cF	4	569 \pm 73 (6)	281 \pm 33 (6)	548 \pm 106 (6)	211 \pm 20 (6)
	cF	6	399 \pm 67 (3)	356 \pm 52 (3)	346 \pm 41 (3)	52 \pm 10 (3)
	cF	8	315 \pm 18 (3)	299 \pm 23 (3)	223 \pm 16 (3)	46 \pm 5 (3)
	M	1	879 \pm 98 (9)	283 \pm 95 (9)	—	—
	M	4	303 \pm 12 (6)	221 \pm 50 (6)	—	—
	cM	1	359 \pm 43 (3)	324 \pm 22 (3)	—	—
	cM	4	562 \pm 58 (3)	333 \pm 52 (3)	—	—
Oestrone (0.3 mg/kg)	cF	1	271 \pm 51 (3)	359 \pm 72 (3)	181 \pm 13 (3)	51 \pm 10 (3)
	cF	2	274 \pm 34 (3)	321 \pm 30 (3)	247 \pm 38 (3)	47 \pm 2 (3)
	cF	4	269 \pm 34 (3)	327 \pm 61 (3)	226 \pm 34 (3)	102 \pm 15 (3)
	cF	6	256 \pm 38 (3)	305 \pm 22 (3)	181 \pm 36 (3)	63 \pm 9 (3)
Testosterone (3.3 mg/kg)	cM	1	271 \pm 50 (3)	172 \pm 14 (3)	—	—
	cM	4	311 \pm 36 (3)	201 \pm 23 (3)	—	—
	F	1	254 \pm 32 (3)	297 \pm 20 (3)	289 \pm 27 (3)	172 \pm 54 (3)
	F	4	272 \pm 40 (3)	343 \pm 48 (3)	326 \pm 19 (3)	193 \pm 35 (3)
	cF	0.5	236 \pm 62 (3)	339 \pm 38 (3)	199 \pm 20 (3)	57 \pm 12 (3)
	cF	1	289 \pm 34 (3)	319 \pm 56 (3)	260 \pm 14 (3)	101 \pm 19 (3)
	cF	2	284 \pm 25 (3)	363 \pm 46 (3)	183 \pm 35 (3)	44 \pm 6 (3)
	cF	4	251 \pm 48 (3)	326 \pm 46 (3)	189 \pm 39 (3)	35 \pm 11 (3)
Testosterone (2 mg/kg)	cF	1	253 \pm 54 (3)	345 \pm 16 (3)	156 \pm 31 (3)	32 \pm 13 (3)
	cF	4	259 \pm 18 (3)	345 \pm 25 (3)	154 \pm 31 (3)	39 \pm 9 (3)
Testosterone (2 mg/kg) + oestrone (1.7 mg/kg)	cF	1	266 \pm 51 (6)	379 \pm 28 (6)	179 \pm 34 (6)	46 \pm 12 (6)
	cF	4	279 \pm 20 (5)	340 \pm 56 (5)	205 \pm 31 (5)	47 \pm 7 (5)
	M	1	261 \pm 20 (3)	314 \pm 31 (3)	—	—
	M	4	295 \pm 78 (3)	323 \pm 64 (3)	—	—

*, †, ‡, see Table 1

uterus were obtained 7 days after injection of ovariectomized mice with chloroform or carbon tetrachloride (Table 3). That these changes were not due to a direct action of the toxic agent on the uterus, but were secondary to the effect on liver, was shown by further experiments in which mice were submitted to partial hepatectomy 3 weeks after ovariectomy, with similar results. With all three methods of treatment, the rise in uterine weight at its greatest was statistically significant ($P < 0.001$). Liver repair is far advanced after this period (Levy *et al.* 1948). In a separate group of six mice, the uteri were examined histologically 9 days after partial hepatectomy. Metoestrus, pro oestrus and, in one case, full oestrus were observed, as compared with dioestrus in ovariectomized controls. It should be noted that not more than 40% of the liver was removed in the partial hepatectomies in the present experiments. Results obtained with mercuric nitrate suggest that changes

in kidney are without effect on uterine weight and glucuronidase activity.

Fractionation of uterine-glucuronidase preparations from ovariectomized mice treated with chloroform and carbon tetrachloride showed all activity to be present in fraction A.

DISCUSSION

The original purpose behind the experiments described above was to decide whether the sites of action of various agents on glucuronidase activity were determined by differences in the properties of the enzyme. No evidence of this was obtained. An increase in β glucuronidase activity appeared to be governed solely by the ability of the agent to stimulate growth processes in the organ in question. Results for uterus did, however, emphasize the need for a preliminary kinetic study with each new organ

Table 3 *Effect of liver regeneration on uterine enzyme and weight*(All values are given as mean \pm s.e., followed (in parentheses) by the number of animals in the group)

Treatment	Sex*	Days after treatment	Total α v /g tissue			Uterine weight (mg)
			Liver†	Kidney‡	Uterus†	
None	F	—	334 \pm 48 (6)	266 \pm 39 (6)	333 \pm 53 (6)	234 \pm 56 (6)
	cF	—	250 \pm 49 (6)	261 \pm 43 (6)	174 \pm 45 (6)	34 \pm 18 (6)
Chloroform (2 g /kg)	F	1	812 \pm 91 (6)	227 \pm 23 (6)	265 \pm 45 (6)	277 \pm 44 (6)
	F	4	696 \pm 61 (3)	238 \pm 27 (3)	342 \pm 34 (3)	372 \pm 36 (3)
	F	7	705 \pm 64 (6)	310 \pm 55 (6)	321 \pm 51 (6)	306 \pm 29 (6)
	cF	1	515 \pm 23 (3)	398 \pm 67 (6)	181 \pm 33 (3)	28 \pm 10 (3)
	cF	4	620 \pm 26 (3)	352 \pm 24 (3)	162 \pm 31 (3)	33 \pm 7 (3)
	cF	7	657 \pm 78 (5)	359 \pm 67 (7)	469 \pm 51 (7)	103 \pm 20 (7)
Chloroform§ (6 g /kg)	cF	8	605 \pm 75 (3)	435 \pm 56 (3)	386 \pm 61 (3)	99 \pm 18 (3)
	cF	10	501 \pm 30 (3)	335 \pm 52 (3)	306 \pm 22 (3)	65 \pm 12 (3)
Carbon tetrachloride (5.3 g /kg)	cF	1	712 \pm 55 (3)	301 \pm 42 (3)	203 \pm 48 (3)	25 \pm 6 (3)
	cF	4	684 \pm 57 (3)	235 \pm 23 (3)	285 \pm 52 (3)	18 \pm 9 (3)
	cF	7	715 \pm 60 (6)	290 \pm 59 (6)	496 \pm 64 (6)	77 \pm 23 (6)
	cF	10	379 \pm 56 (3)	292 \pm 12 (3)	205 \pm 20 (3)	42 \pm 5 (3)
Partial hepatectomy	cF	2	572 \pm 53 (3)	284 \pm 22 (3)	225 \pm 67 (3)	75 \pm 14 (3)
	cF	4	532 \pm 83 (3)	305 \pm 29 (3)	240 \pm 42 (3)	64 \pm 8 (3)
	cF	6	625 \pm 89 (3)	342 \pm 34 (3)	412 \pm 31 (3)	123 \pm 14 (3)
	cF	8	535 \pm 61 (6)	328 \pm 54 (6)	395 \pm 43 (6)	115 \pm 19 (6)
	cF	12	424 \pm 41 (3)	329 \pm 32 (3)	277 \pm 29 (3)	53 \pm 6 (3)
Mercuric nitrate (20 mg /kg)	cF	3	241 \pm 32 (3)	523 \pm 27 (3)	179 \pm 18 (3)	30 \pm 4 (3)
	cF	6	238 \pm 26 (3)	323 \pm 24 (3)	168 \pm 26 (3)	39 \pm 6 (3)

*, †, ‡, see Table 1

§ Divided into three daily doses of 2 g /kg, timed from first injection

examined for glucuronidase activity. The choice of pH 5.2 for determining activities in liver or kidney with phenylglucuronide would seem to be justified for most purposes.

Changes in the susceptibility of the mouse kidney to chloroform necrosis were faithfully reflected in the figures for glucuronidase activity. The value of such figures as a biochemical index of growth is illustrated by the discovery of new facts relating to liver and uterus. The action of oestrone on liver in normal and castrate males and in ovariectomized females, and the antagonizing of this action by testosterone are in accordance with the view of Bullough (1946) that the effects of such hormones are more widespread throughout the body than is generally appreciated. The absence of any action by oestrone on liver in normal females suggests some form of control of this organ by the ovary.

The changes observed in uterus during liver regeneration in the ovariectomized mouse can only be explained on the assumption that the body is capable of producing an extra ovarian growth hormone for uterus in significant amounts. In this case, there is obvious need for care in interpreting certain experiments (Talbot, 1939; Pincus & Martin, 1940; Segaloff, 1946; Roberts & Szego, 1947) in which the action of liver damage and regeneration in enhancing the effectiveness of administered or ovarian oestrogens is claimed to be due to 'depressed inactivation' or 'accelerated activation' of the hormones. In view of the effect of oestrone on the

liver, a hitherto unsuspected complication must be looked for in the action of this compound on the uterus of the ovariectomized mouse.

SUMMARY

1 Both β glucuronidase fractions found by Mills (1947) in ox spleen are present in mouse liver and kidney, whilst the uterus contains only one of these.

2 The two glucuronidase fractions in liver and kidney respond identically to agents causing changes in the enzyme activity.

3 In the uterus, as in other organs, changes in glucuronidase activity reflect changes in growth, and the action of oestrone on the enzyme is antagonized by testosterone.

4 Oestrone produces marked increases in glucuronidase activity and cell division in the liver in ovariectomized mice. This action, which is also seen in normal and castrate males, but not in normal females, is antagonized by testosterone.

5 During liver regeneration following chloroform or carbon tetrachloride poisoning or partial hepatectomy, uterine weight and glucuronidase activity increase in ovariectomized mice in absence of administered oestrogen.

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Concentration of Lipids in the Brain of Infants and Adults

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Histologically it has been shown that the fibre tracts of the brain are not fully myelinated at birth, but that myelination is completed later, coincident with the functional development of the central nervous system. Most of the medullated fibres of the nervous system are in the white matter, whereas most of the bodies of the nerve cells are in the grey matter. In order to investigate, therefore, the lipid components of the myelin sheath, the lipid distribution in the grey matter and white matter of the brain of the newborn infant has been compared with that of the adult brain.

The important lipids of the central nervous system are cerebrosides, cholesterol and the phospholipins, lecithin, sphingomyelin and kephalin. In a previous report (Johnson, McNabb & Rossiter, 1948a) it was shown that white matter of brain is distinguished from grey matter by a greater concentration of cerebroside, free cholesterol and sphingomyelin. It was suggested that these lipids, rather than lecithin and kephalin, formed the lipid components of 'myelin'. Additional evidence was obtained for this view when it was found that medullated peripheral nerve was relatively rich in cerebroside, cholesterol and sphingomyelin, thus resembling the white matter of brain rather than grey matter (Johnson, McNabb & Rossiter, 1948b). It has now been shown that white matter of adult brain, in which myelination is complete, differs from 'white matter' of newborn infant brain in that it contains a higher concentration of these same lipids (cerebroside, cholesterol and sphingomyelin).

METHODS

The brain was removed from each of five infants and five adults as soon as possible after death. The infants, whose ages ranged from 7 months' gestation (premature) to full term, died at birth or shortly afterwards. Samples of both grey matter and 'white' matter were taken from the cerebral hemispheres. The grey matter consisted of a thin shaving from the surface of the cerebrum. As there was practically no visible white matter in the infant, the 'white' sample was taken from the positions where white matter was known to occur in the adult brain.

A sample of tissue (1-3 g) was rapidly weighed and repeatedly extracted with 50 ml portions of a 1:1 ethanol ether mixture as described previously (Johnson *et al* 1948a). Additional samples of both grey matter and white matter were taken for the determination of the wet-weight to dry weight ratio. A portion of the fresh tissue was added to a tared covered crucible, the crucible and tissue weighed, dried in an oven at 105° for 24 hr, cooled in a desiccator and reweighed. From these figures, the water content of the tissue was calculated. The concentrations of cerebroside, free cholesterol, total cholesterol, total phospholipin, mono aminophospholipin and lecithin were determined in samples of the extract as previously described (Johnson *et al* 1948a), and from these figures were calculated the concentrations of ester cholesterol, sphingomyelin and kephalin.

RESULTS

The figures for both white and grey matter of adult brain (Table 1) were similar to those obtained previously, when two human brains only were analyzed (Johnson *et al* 1948a), and were considerably greater than those for infant brain, chiefly

Table 1 *Concentration of lipids in grey and white matter of infant and adult brain, expressed as percentage of fresh tissue weight*(Five infant and five adult brains examined Results given as mean \pm S.E.M.)

	Grey matter			White matter		
	Infant (%)	Adult (%)	Adult/infant ratio	Infant (%)	Adult (%)	Adult/infant ratio
Cerebroside	0.52 \pm 0.06	0.87 \pm 0.24	1.67 1	0.58 \pm 0.01	4.78 \pm 0.19	8.25 1
Total cholesterol	0.49 \pm 0.06	0.99 \pm 0.02	2.02 1	0.67 \pm 0.07	4.22 \pm 0.17	6.30 1
Free cholesterol	0.48 \pm 0.07	0.97 \pm 0.05	1.98 1	0.64 \pm 0.07	4.15 \pm 0.18	6.50 1
Ester cholesterol	0.01 \pm 0.00	0.02 \pm 0.00	—	0.03 \pm 0.03	0.07 \pm 0.07	—
Total phospholipin	1.89 \pm 0.27	3.36 \pm 0.05	1.78 1	2.09 \pm 0.19	7.03 \pm 0.29	3.36 1
Monoaminophospholipin	1.77 \pm 0.26	2.83 \pm 0.08	1.60 1	1.96 \pm 0.20	5.03 \pm 0.37	2.57 1
Lecithin	0.75 \pm 0.08	0.99 \pm 0.09	1.32 1	0.85 \pm 0.07	1.37 \pm 0.12	1.61 1
Sphingomyelin	0.12 \pm 0.05	0.53 \pm 0.04	4.42 1	0.13 \pm 0.04	2.00 \pm 0.18	15.40 1
Kephalin	1.02 \pm 0.18	1.85 \pm 0.04	1.81 1	1.11 \pm 0.13	3.66 \pm 0.27	3.30 1

because the water content of adult brain was much less than that of infant brain.

Nevertheless, from Table 1, it can be seen that the ratio of the concentration of each individual lipid in the grey matter of the adult to the concentration of the same lipid in the infant was usually less than 2 and never greater than 4.42. For white matter the ratio for cerebroside, cholesterol (almost all of which was in the free form) and sphingomyelin was much greater, suggesting that it was a greater concentration of these lipids that distinguished the white matter of the adult brain from that of the infant.

The results of Table 1 appear somewhat different after allowance has been made for the water content of the tissues. Table 2 shows that the water content of infant brain was much higher than that of adult brain, and also that the value for grey matter of infant brain was not significantly different from that for white matter. The water content of the adult brain was much less, and it was found that grey matter had more than twice the water content of white matter. Thus, as the brain developed and myelin was deposited, the whole organ lost water, but relatively more was lost from the white matter than from the grey matter.

In Table 3 the values for the lipid components of both grey and white matter of infant and adult brain

are given in terms of dry weight. It can now be seen how strikingly similar was the distribution of lipids in the grey matter of the infant brain to that in the adult. For grey matter, with the exception of sphingomyelin with a ratio of 2.33, the adult to infant

Table 2 *Water content of grey and white matter of infant and adult brain*(Five infant and five adult brains examined Results given as mean \pm S.E.M.)

	Grey matter (mg/mg dry tissue)	White matter (mg/mg dry tissue)
Infant	9.74 \pm 0.69	9.82 \pm 0.72
Adult	5.34 \pm 0.10	2.40 \pm 0.06

ratio for each individual lipid was between 0.80 and 1.23. For white matter, however, the picture was quite different. The ratio for cerebroside and free cholesterol was 2.10 or more, and for sphingomyelin it was 4.87. Thus the white matter of adult brain had a higher concentration of cerebroside, free cholesterol and sphingomyelin than had the white matter of infant brain. The concentrations of total phospholipin in the white matter of the adult brain and infant brain did not differ significantly. There was no difference in the concentration of kephalin, and the greater concentration of sphingomyelin in the white

Table 3 *Concentration of lipids in grey and white matter of infant and adult brain, expressed as percentage of dry tissue weight*(Five infant and five adult brains examined Results given as mean \pm S.E.M.)

	Grey matter			White matter		
	Infant (%)	Adult (%)	Adult/infant ratio	Infant (%)	Adult (%)	Adult/infant ratio
Cerebroside	5.64 \pm 0.91	5.54 \pm 0.80	0.98 1	6.21 \pm 0.39	16.28 \pm 0.99	2.61 1
Total cholesterol	5.09 \pm 0.37	6.28 \pm 0.14	1.23 1	7.00 \pm 0.44	14.33 \pm 0.56	2.04 1
Free cholesterol	5.03 \pm 0.35	6.17 \pm 0.20	1.23 1	6.70 \pm 0.33	14.08 \pm 0.55	2.10 1
Ester cholesterol	0.06 \pm 0.04	0.10 \pm 0.05	—	0.30 \pm 0.07	0.26 \pm 0.22	—
Total phospholipin	19.56 \pm 1.39	21.27 \pm 0.49	1.08 1	22.04 \pm 0.59	23.84 \pm 0.73	1.08 1
Monoaminophospholipin	18.20 \pm 1.36	17.96 \pm 0.59	0.98 1	20.64 \pm 0.62	17.01 \pm 1.15	0.82 1
Lecithin	7.81 \pm 0.45	6.25 \pm 0.63	0.80 1	9.07 \pm 0.28	4.63 \pm 0.34	0.51 1
Sphingomyelin	1.30 \pm 0.46	3.03 \pm 0.29	2.33 1	1.40 \pm 0.58	6.82 \pm 0.67	4.87 1
Kephalin	10.46 \pm 1.08	11.71 \pm 0.40	1.11 1	11.57 \pm 0.58	12.39 \pm 0.83	1.07 1

matter of the adult brain was balanced by a lesser concentration of lecithin (Table 3). This point is well illustrated in Table 4, where the individual phospholipins are expressed as a percentage of the total phospholipin. For white matter the percentage of kephalin was about the same (52 %) for both infant and adult brain. Lecithin accounted for 41 % of the total phospholipin for infant brain and only 19 % for the adult, whereas sphingomyelin accounted for 6 % of the total phospholipin for infant brain and 29 % for the adult.

by the numerous publications of Donaldson (see Donaldson & Hatai, 1931, for references). This raises the question of whether infant brain should be compared with adult brain on a wet weight basis, as has been done by many workers. For instance, Backlin (1930) found that the concentration of total phospholipin in the whole brain of the adult rabbit was greater than that of a newborn rabbit in terms of wet weight, but that there was no difference when dry weight was used as a reference standard. We have found the same to be true for the white

Table 4 *Concentration of phospholipins in grey and white matter of infant and adult brain, expressed as percentage of total phospholipid*

(Five infant and five adult brains examined. Results given as mean \pm S.E.M.)

	Grey matter			White matter		
	Infant (%)	Adult (%)	Adult/infant ratio	Infant (%)	Adult (%)	Adult/infant ratio
Lecithin	40.28 \pm 2.31	29.16 \pm 2.46	0.72 : 1	41.22 \pm 1.85	19.42 \pm 1.35	0.46 : 1
Sphingomyelin	6.62 \pm 2.37	15.68 \pm 1.40	2.37 : 1	6.28 \pm 2.61	28.80 \pm 3.31	4.57 : 1
Kephalin	53.10 \pm 2.48	55.20 \pm 2.55	1.04 : 1	52.50 \pm 1.94	51.78 \pm 2.17	0.99 : 1

DISCUSSION

Frankel & Lannert (1910) found that the concentration of total lipid in the whole brain of the adult was greater than that in the brain of the newborn infant. Using the classical differential solubility techniques they showed that the increase in lipid concentration in adult brain was not confined to one lipid fraction only. This work was confirmed by Schiff & Strinsky (1921) and recently by Schuwurth (1940). Raske (1886) found that the whole brain of foetal calves contained no cerebroside, a result confirmed by Mendel & Leavenworth (1908) using pigs. Noll (1899), when studying the 'protagon' fraction of human brain, observed that with the beginning of myelination there appeared an ethanol soluble substance from which a reducing sugar was split off on hydrolysis. Koch & Koch (1913) showed that cerebroside was not present in the brain of newborn rats, but that the concentration of cerebroside, phospholipin and cholesterol increased with age. A similar finding was reported for the dog (Smith & Mair, 1912-13a), the rabbit (Backlin, 1930) and man (Koch & Mann, 1907; MacArthur & Doisy, 1919). Less comprehensive work on total phospholipin has been done for rat brain (Lang, 1937; Fries, Entenman, Changus & Chalkoff, 1941), and human brain (Bergamini, 1925; Singer & Deutschberger, 1928; Cattaneo, 1932). Similar studies on total cholesterol have been reported for rat brain (Lang, 1937; Fries *et al.* 1941), dog brain (Mansfeld & Liptak, 1913) and human brain (Rosenheim, 1914; Bergamini, 1925; Page & Menschick, 1931; Cattaneo, 1931). In addition, McConnell & Sinclair (1937) using rats, reported that the lecithin and kephalin fatty acids increased during growth. They did not estimate the individual phospholipins, nor did they distinguish between white and grey matter, hence from these studies very little can be deduced about the chemical nature of myelin.

That foetal brain contains more water than does adult brain was known to most of the above authors and the subject has been well reviewed for the rat

matter of human brain. In terms of wet weight (Table 1) the concentration of total phospholipin was greater in the adult than in the newborn infant, but when referred to unit dry weight there was no such difference (Table 3). Previously (Johnson *et al.* 1948a, b), lipids have been expressed as a percentage of 'essential lipid', i.e. as a percentage of the sum of the cerebroside, total cholesterol and total phospholipin. The values for the adult and infant brains were also calculated on an 'essential lipid' basis, but this method provided no further information than that already given in Table 3.

The recent report of Williams, Galbraith, Kaucher, Moyer, Richards & Macy (1945) on the lipids of the brains of rats of different ages is of interest for they estimated the individual phospholipins. It is difficult, however, to make a strict comparison of their results with our own, for, besides the species difference, the method which they used to distinguish the individual phospholipins was not the same as ours, and the white and grey matter were not separated. Williams *et al.* (1945) found that with increasing age, in addition to the well established greater concentrations of phospholipin, cerebroside and cholesterol, there was a large increase in kephalin, a lesser increase in sphingomyelin and a decrease in lecithin. In our study on the white matter of the human brain there was a lesser concentration of lecithin in the adult than in the infant, the same concentration of kephalin and a much greater concentration of sphingomyelin.

Many of the pioneers of brain chemistry noted the great difference between the lipids of white and those of grey matter. For instance, both Petrowsky (1873) and Thudichum (1901) found considerably more cholesterol and

cerebroside in white than in grey matter, and less lecithin. These findings have subsequently been confirmed (Smith & Mair, 1912-13b, Frankel & Linnert, 1910, Kirschbaum & Linnert, 1912, Yasuda, 1937, Randall, 1938, Johnson *et al* 1948a). In addition, Schmidt, Benotti, Hershman & Thannhauser (1946) showed that the concentration of sphingomyelin in the white matter of ox brain was greater than that in grey matter. Many of these workers also noted that the concentration of water was greater in grey than in white matter.

That the lipids of white matter of foetal brain resemble those of grey matter was first pointed out by Raske (1886) and later by Frankel & Linnert (1910) and Smith & Mair (1912-13b). Not only have we confirmed this similarity for cerebroside, cholesterol and total phospholipin, but also for lecithin, sphingomyelin and kephalin.

Since the white matter of adult brain is distinguished from that of the brain of the newborn by greater concentrations of cerebroside, cholesterol and sphingomyelin, and, since it has been shown histologically that myelination of the various tracts is complete in the white matter of the adult brain and not in the brain of the newborn infant, it is perhaps reasonable to assume that these are the principal lipids of the myelin sheath. However, a word of caution is necessary. Waelsch, Sperry & Stoyanoff (1941) have pointed out that after birth lipid is deposited in the brain of the rat as a result of two processes, (a) growth and (b) myelination. Immediately after birth and before myelination is complete there is an active deposition of brain lipids as shown by isotope studies. The incorporation of radioactive phosphorus in the phospholipin fraction (Fries, Changus & Chaikoff, 1940) and of deuterium in both the fatty acid and non saponifiable fraction of the brain lipids (Waelsch *et al* 1941) was greatest in young rats immediately after birth, when growth was greatest, and decreased sharply with increasing age, even although myelination was proceeding rapidly. Such experiments show that much new lipid material is deposited in the brain after birth as a result of growth, and the differences between the adult and the newborn need not be necessarily the result of myelination.

In general, the lipid distribution is similar in both white and grey matter of infant brain and closely resembles that of grey matter of adult brain. The white matter of the adult is distinguished from that of the newborn by a much greater concentration of cerebroside, free cholesterol and sphingomyelin. This observation, together with the finding that these lipids are present in high concentrations in peripheral medullated nerves, makes it likely that cerebroside, free cholesterol and sphingomyelin, rather than lecithin and kephalin, go to make up the lipids of 'myelin'.

SUMMARY

1 The concentration of cerebroside, free cholesterol, total cholesterol, total phospholipin, lecithin, sphingomyelin and kephalin has been determined in both the grey and white matter of the brains of five infants and five adults.

2 The water content of both white and grey matter of infant brain was greater than that of adult brain, and for the adult the water content of grey matter was much greater than that of white matter.

3 The distribution of lipids in the white matter of infant brain resembled that of the grey matter and also the grey matter of adult brain.

4 The distribution of lipids in the white matter of adult brain differed from that of the white matter of infant brain in that there was a higher concentration of cerebroside, free cholesterol and sphingomyelin.

5 Although, in terms of dry weight, the white matter of adult brain had about the same total phospholipin content as white matter of infant brain, it contained more sphingomyelin and less lecithin.

6 The data reported here, taken in conjunction with those of previous studies, suggest that cerebroside, free cholesterol and sphingomyelin are the principal lipid components of the 'myelin' sheath of nerve fibres.

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Effects of Amidines on Oxidases of *Escherichia coli* and of Animal Tissues

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Interest in the diamidines as antiprotozoal and antibacterial agents led us to consider their possible mechanism of action, and a study of their effects on oxidation systems offered a method of approach. Bernheim (1943, 1944) reported that certain amidines inhibited the oxidation of D-proline and of both isomers of alanine by *Escherichia coli*, but were without effect on the oxidation of glucose, succinate or pyruvate at similar concentrations.

We considered that further valuable information would possibly be revealed by studying the effects of amidines at higher concentrations, using also a wider range of substrates. Accordingly, we have carried out an investigation along these lines, with *Esch. coli* as the test organism. Propamidine (1,3-di-(4'-amidinophenoxy)propane) was mainly employed, but a number of experiments were also carried out with hexamidine (1,6-di-(4'-amidinophenoxy)hexane) and two halogenated derivatives, i.e. dibromopropamidine (1,3-di-(2'-bromo-4'-amidinophenoxy)propane) and moniodohexamidine (1,4'-amidinophenoxy-6-(2'-iodo-4'-amidinophenoxy)-hexane). Some parallel experiments on the oxidation systems of rat tissues were included. A recent communication (Wien, Harrison & Freeman, 1948), in which some of our results were quoted, has dealt with the diamidines as bactericides.

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EXPERIMENTAL

Measurement of respiration

The Warburg manometric technique was used for following O_2 uptakes at 37°. The bacterial suspension or tissue preparation was contained in the main vessel in 0.033M phosphate buffer (for tissue preparations, phosphate Locke medium was used), with or without the amidine salt in appropriate concentration. A 6% KOH solution (0.2 ml) was introduced into the central tube of the vessel, and soaked into a small piece of rolled Whatman no. 41 filter paper. The substrate solution (0.2 ml.) was contained in the side arm and was tipped into the main vessel after a suitable equilibration or incubation period. The total volume of vessel contents, including those of the side arm and central tube, was always 3 ml.

Organisms

Esch. coli (National Collection of Type Cultures no. 4144) was used. The organisms were sown on tryptic digest-agar plates, and after 18-24 hr growth the cultures were washed off the plates, using 3 ml of distilled water for each plate. The collected suspension was centrifuged, was twice washed by resuspending in distilled water and recentrifuging, and finally diluted to 7.15 mg dry wt./ml. by the use of Wellcome standard opacity tubes. Of this suspension 0.5 ml was used for each vessel. It was confirmed that no appreciable change in the suspension occurred during the time occupied by the experiments. The O_2 uptake due to the organisms alone, without substrate, was usually not greater than 20 μ l/hr, since this was small in comparison with the O_2 uptake in the presence of most substrates employed, it is permissible to regard the difference as representative of the O_2 used in the

oxidation of the substrate. Experiments were carried out at pH values of 5.6, 6.8, 7.4 and 7.8, in view of Bernheim's (1944) emphasis on pH.

Tissue preparations

Preparations were made from the organs of rats in the minimum possible time after killing.

Liver was ground with alundum and 3–4 ml 0.9% NaCl to a fine suspension, filtered by squeezing through gauze, and the tissue washed by diluting to 10 ml with 0.9% NaCl. After centrifuging at high speed the deposit was suspended in 0.9% NaCl (5 ml/g original tissue) and 0.5 ml used in each vessel for studying the activities of the succinate, choline and cytochrome oxidase systems.

Kidneys were minced in 0.9% NaCl to give a fine suspension, which was washed by centrifuging and diluted with 0.9% NaCl (5 ml/g original tissue). For each vessel, 0.5 ml was used for studying D amino acid oxidase activity.

Brain The whole brain was made to a uniform suspension with 3–4 ml 0.9% NaCl in a hard glass homogenizer, and diluted with 0.9% NaCl (6 ml homogenate/g original tissue). Of this 1 ml was used for each experiment with the lactate and glucose oxidase systems.

Amidine derivatives

These were used as double isethionate salts.

RESULTS

Effects of amidines on oxidases of *Escherichia coli*

The results of a series of experiments with a number of substrates at different pH values are shown in Table 1. No initial incubation period was allowed in

after 2 hr incubation at pH 7.8, and these effects may be compared with those observed by Bernheim (1944) with alanine and proline. With lactate, inhibitions of roughly the same order were brought about by dibromopropamidine, hexamidine and moniodohexamidine.

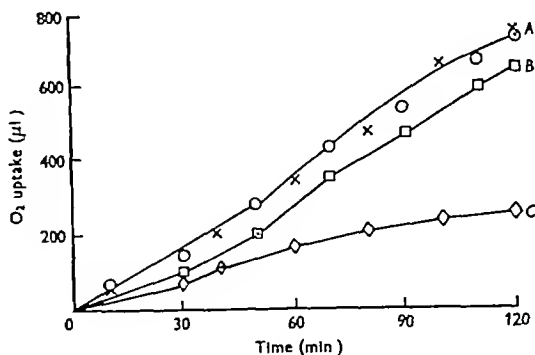


Fig. 1. Effects of propamidine on Na lactate oxidation by *Esch. coli*. Bacterial suspensions at 37° with 0.01M-Na lactate. A, O pH 5.6; B, × with 12.5×10^{-5} M-propamidine, pH 5.6; C, with 12.5×10^{-5} M propamidine, pH 7.8.

Table 2 gives the results of a series of experiments in which the amidines were incubated with the bacterial suspension at pH 7.8 for 2 hr at 37° before the addition of substrate. Q_{O_2} values (μ l O_2 absorbed/mg dry wt organisms/hr) are included. Inhibitions by particular amidines on different substrates can be compared. Oxidation of all the substrates (malate, succinate and glutamate in addition to

Table 1. Effects of amidines on oxidations by *Escherichia coli*

(Washed suspensions of bacteria, 37°, substrate added at the same time as amidine.)

Substrate (0.01M)	Amidine (12.5×10^{-5} M)	Inhibitions (%)							
		pH 5.6		pH 6.8		pH 7.4		pH 7.8	
		In 1st hr	In 2nd hr	In 1st hr	In 2nd hr	In 1st hr	In 2nd hr	In 1st hr	In 2nd hr
Na lactate	Propamidine	8	0	23	35	38	57	39	78
Na lactate	Hexamidine	0	0	30	51	39	61	63	76
Na lactate	Moniodohexamidine	25	24	45	52	66	84	73	90
Na pyruvate	Propamidine	0	0	0	19	30	32	33	21
Glucose	Propamidine	0	0	0	0	31	54	28	65
Na acetate	Propamidine	0	2	0	12	6	45	40	78
D Alanine	Propamidine	15	44	25	50	21	59	53	73

these experiments, beyond that required for thermal equilibration. Fig. 1 illustrates the variations of oxygen uptake with time, in the case of lactate, and the manner in which this is influenced by propamidine at pH values of 5.6 and 7.8, similar variations with time were observed with the other substrates. The systems governing the oxidations of lactate, glucose, pyruvate, acetate and D alanine were all substantially inhibited. The inhibitions were maximal

those mentioned in Table 1) were inhibited by concentrations of amidines of the order of 12.5×10^{-5} M or less. Lactate was affected to approximately the same degree as alanine, both these systems were sensitive to concentrations of propamidine as low as 1.25×10^{-5} M. The halogenated derivatives inhibited oxygen uptake slightly more than the parent compounds, and hexamidine rather more than propamidine, but the differences were small.

Table 2 *Effects of amidines on oxidation systems of Escherichia coli*

(Washed suspensions of bacteria, incubated 2 hr with amidine before tipping in substrate solution, reaction at 37°, pH 7.8)

Substrate (0.01 M)	Amidine, concentration ($\text{M} \times 10^{-5}$)	Q_{O_2} Control without amidine	Q_{O_2} With amidine	Inhibition by amidine (%)
D Alanine	Propamidine, 1.25	30.5	20.4	33
D Alanine	Propamidine, 12.5	30.5	6.2	80
D Alanine	Hexamidine, 12.5	33.2	2.0	94
D Alanine	Dibromopropamidine, 12.5	33.2	4.2	87
Na lactate	Propamidine, 1.25	94.0	57.6	39
Na lactate	Propamidine, 12.5	94.0	30.0	68
Na lactate	Hexamidine, 12.5	94.6	22.4	76
Na lactate	Dibromopropamidine, 12.5	55.8	9.2	84
Na lactate	Monoiodohexamidine, 12.5	69.0	6.2	91
Glucose	Propamidine, 12.5	31.6	13.2	58
Glucose	Dibromopropamidine, 12.5	62.4	9.2	86
Glucose	Monoiodohexamidine, 12.5	77.0	0.6	99
Na malate	Propamidine, 12.5	26.2	7.2	72
Na malate	Hexamidine, 12.5	35.2	4.0	89
Na malate	Dibromopropamidine, 12.5	13.4	1.6	88
Na malate	Monoiodohexamidine, 12.5	39.6	3.0	92
Na acetate	Propamidine, 12.5	64.2	9.2	86
Na succinate	Propamidine, 12.5	34.6	20.0	42
Na glutamate	Propamidine, 12.5	14.6	4.4	69
Na pyruvate	Propamidine, 12.5	74.8	23.8	68

Table 3 *Effects of propamidine on tissue oxidation (37° and pH 7.6)*

Source of enzyme	Substrate, concentration (M)	Propamidine ($\times 12.5 \times 10^{-5}$ M)	Period of in- cubation of enzyme and amidine (hr)	Duration* of experi- ment (hr)	O_2 uptake (μl)	Q_{O_2}	Inhibition (%)
Rat-liver extract	Nil	0	2	1	2.7	0.06	—
	Na succinate, 0.03	0	2	1	333	8.32	—
	Na succinate, 0.03	1	2	1	313	7.82	6
	Choline chloride, 0.03	0	2	1	77	1.92	—
	Choline chloride, 0.03	1	2	1	23	0.58	73
	Choline chloride, 0.03	0	Nil	1	184	4.60	—
	Choline chloride, 0.03	1	Nil	1	48	1.20	74
	p-Phenylenediamine, 0.5	0	2	1	209	3.22	—
	p-Phenylenediamine, 0.5	1	2	1	233	5.84	0
Minced rat kidney	Nil	0	Nil	1	38	0.96	—
	D Alanine, 0.013	0	Nil	1	169	4.22	—
	D Alanine, 0.013	1	Nil	1	167	4.18	<1
Brain homogenate	Nil	0	1	2	66	0.50	—
	Na lactate, 0.017	0	1	2	165	1.23	—
	Na lactate, 0.017	1	1	2	149	1.11	10
	Glucose, 0.013	0	1	2	153	1.14	—
	Glucose, 0.013	1	1	2	131	0.97	14

* After tipping in substrate

Effects of amidines on tissue oxidases

Effects of propamidine on some oxidation systems of rat tissues are shown in Table 3, the concentrations of the foregoing experiments were used. No significant effects were exerted on oxidation of glucose and lactate by rat brain, the succinate and cytochrome oxidases (the latter represented by the oxidation of p-phenylenediamine) and the D-amino acid oxidase activity of kidney were likewise unaffected. The only

positive effect noted was that on choline oxidase, which was appreciably inhibited by 12.5×10^{-5} M-propamidine.

DISCUSSION

The observed effects of the amidines on the oxidases of *Esch. coli* are of interest with reference to the antibacterial actions of these compounds. Although no definite conclusions regarding the mode of inhibition of bacterial growth may be drawn from results of

enzyme studies on only one organism, the ability of amidines to exert toxic effects on different oxidation systems is a factor to be taken into account, especially in view of the fact that a high pH value favours inhibition of growth (Elson, 1945) as well as of respiration. The connexion is not apparently a simple one, since the differences in bacteriostatic power between halogenated derivatives and parent compounds are not paralleled by the same differences in anti oxidase activity. To give an example, the concentrations required to produce bacteriostasis for *Esch coli* are $11.8 \times 10^{-5} \text{ M}$ for propamidine and $0.57 \times 10^{-5} \text{ M}$ for dibromopropamidine (Wien *et al* 1948) the corresponding figures for inhibition of oxidation were 80 and 87 % for alanine, and 68 and 84 % for lactate, using $12.5 \times 10^{-5} \text{ M}$ concentrations of the amidines. It cannot, therefore, be claimed that the anti oxidase effects reflect the therapeutic efficacy of the compounds.

Regarding the mechanism of the inhibitory effect on respiration, it may be that the amidines exert effects on the particular dehydrogenases concerned with the different substrates, but the fact that so many systems are involved would appear to render this unlikely. It is more likely that an inhibitory effect is exerted on a respiratory mediator common to the various systems. One interesting possibility is that the system governing the direct transfer of molecular oxygen may be depressed, this would partly be supported by the absence of effect on the lactate oxidation system of brain, a cozymase linked system, and by the slight effects of the amidines in inhibiting the growth of anaerobic organisms. It is conceivable that the cytochrome system peculiar to

Esch coli may be inhibited, the absence of an effect on the cytochrome oxidase of liver does not conflict with this possibility, in view of the different cytochrome compositions of the two types of cell (Keilin & Harpley, 1941). It was not possible to pursue the point.

Finally, experiments on tissue oxidation revealed an apparently specific inhibition of the choline oxidase of liver. This point is of interest in connexion with the chronic toxicity of the amidines, which produce characteristic fatty changes in the liver. In view of the part played by choline metabolism in liver function, especially in regard to fatty changes, the inhibitory effect of propamidine on choline oxidase *in vitro* may well have some bearing on the degenerative changes produced in the liver *in vivo*.

SUMMARY

1 The respiration of washed cells of *Escherichia coli* in presence of various substrates was strongly inhibited by certain diamidines in concentrations of the order of $1.25\text{--}12.5 \times 10^{-5} \text{ M}$.

2 There was no simple relationship between the bacteriostatic potencies of the amidines and their effects on respiration.

3 Propamidine had no effect on several tissue oxidation systems, but inhibited the choline oxidase of liver.

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The Quantitative Determination of Barbiturates in Tissues by Ultraviolet Absorption Spectrophotometry

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The purpose of this paper is to describe a method for the estimation of very small amounts of barbiturates in mammalian tissues. The problem arose in connexion with work on the intermediary meta-

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bolism of the drugs, when it was found that the methods previously described were inapplicable for various reasons which will be discussed.

The basis for the various spectrophotometric procedures is the fact that barbituric acid and its derivatives of pharmacological importance, including

barbital (5,5-diethylbarbituric acid) and pentobarbital (5-ethyl-5,2'-pentylbarbituric acid) possess in alkaline solution characteristic absorption bands in the ultraviolet. Changes in the location and intensity of these bands in relation to variations in molecular structure have been discussed by Loofbourow & Stimson (1940), Stuckey (1940, 1941, 1942), Klotz & Askounis (1947), and Walker, Fisher & McHugh (1948). The facts relevant to the present method are (a) from c pH 6.5–c pH 10, a well-defined absorption maximum occurs at a wave length of 239 m μ , (b) as the reaction becomes still more alkaline, the position of the maximum shifts in the direction of longer wave lengths, (c) in alkaline solution, the extinction at 239 m μ is directly proportional to the concentration of barbiturate, in accordance with the Lambert Beer law (Fig 1). On the basis, therefore, of spectrophotometric data the concentration of the drugs can easily be determined in pure solutions.

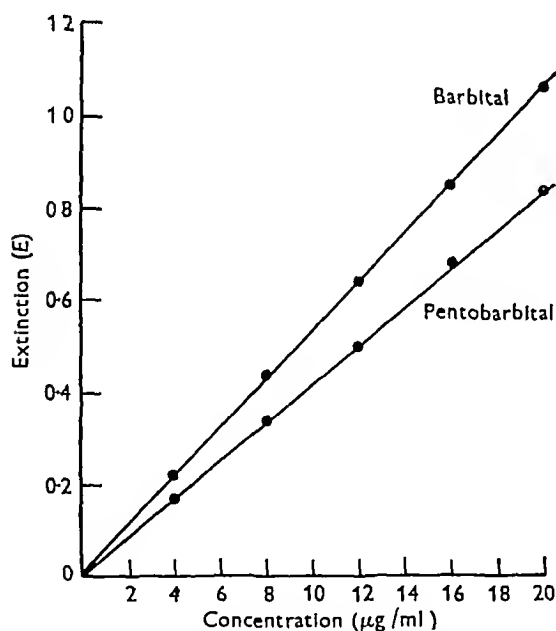


Fig 1 Relation of extinction to concentrations of barbital and pentobarbital in alkaline solutions, pH 9.4, wave length 239 m μ

In attempts made to recover barbiturate from tissues, however, other substances are usually extracted at the same time. Some of them absorb radiation in the same region of the ultraviolet as the barbiturates. It has not been practicable to recover the drugs quantitatively in 'pure' solution, and it is therefore evident that any spectrophotometric method for the estimation of barbiturate in the presence of such impurities is valueless unless it allows for the contribution made by these interfering substances to the total absorption.

The present method achieves this by making use of the observation that the intensities of absorption at 239 m μ of barbiturate and of the interfering substances differ in their dependence on pH. The extinction of barbiturate is characteristically related to pH, being maximal at pH 10 and decreasing steeply towards the acid, less steeply towards the alkaline side (Fig 2). The ratio of the extinctions at any two values of pH is independent of concentration.

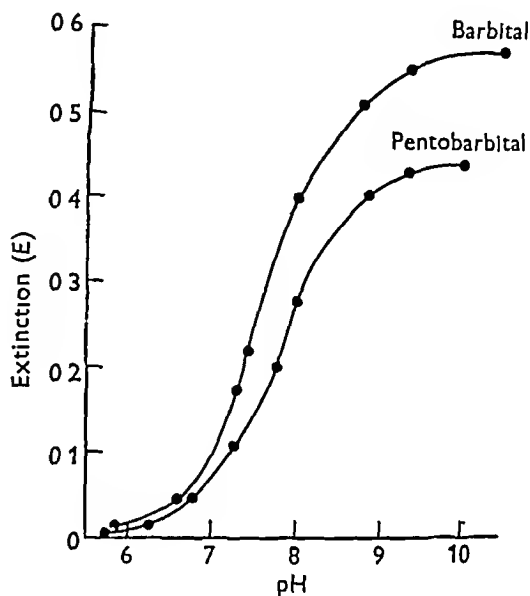


Fig 2 Relation of extinction to pH for barbital and pentobarbital, concentration of drugs 10 μg/ml, wave length 239 m μ

In contrast, the extinction due to the interfering substances obtained from different tissues by the present procedure has been shown to be practically constant over certain ranges of pH. Preliminary experiments are necessary to determine these ranges and the change in extinction of barbiturate when the pH is altered to the same extent. The results permit calculation of the concentration of barbiturate in impure solutions of this nature.

METHOD

Principle The tissue is homogenized and the proteins are precipitated by ethanol. The barbiturate in the acidified protein free filtrate is extracted with ether and passed into alkali. To two samples of this alkaline solution are added phosphate or borate solutions which bring the pH to different known values. The extinctions at 239 m μ of the resulting solutions are measured, and from these the concentration of barbiturate in the tissue can be determined by calculation.

Apparatus The extinction measurements were made with a Beckman Model DU photoelectric spectrophotometer with 1 cm quartz cells. pH was determined electrometrically with a glass electrode.

Reagents Conc HCl (A R), absolute ethanol, diethyl ether (A R), NaCl (A R), NaOH solution 0.2M, KH_2PO_4 and H_3BO_3 KCl solutions as for the preparation of buffer solutions according to Vogel (1944), except that the concentrations of the stock phosphate and borate solutions are 1.0M and 0.5M respectively

Experimental procedure

Preliminary experiments About 1 g of normal tissue is extracted as described below. The extracted interfering substances are highly concentrated in the 5 ml of alkaline solution obtained, this is therefore made up to 50 ml with more 0.2M-NaOH solution, and 5 ml amounts of this are then pipetted into a series of test tubes graduated at 10 ml.

Into another series of test tubes similarly graduated are put 5 ml volumes of a 0.002% (w/v) solution of the barbiturate in 0.2M aqueous NaOH (equivalent to 100 μg drug/tube). Into a third series of test tubes are put 5 ml volumes of pure 0.2M NaOH solution.

To corresponding tubes of all three series are added increasing volumes of the phosphate or borate solutions, so that the pH increases in each series by steps of approx 0.5 of a unit from 6 to 10. The quantities to be added may be calculated from the tables given by Vogel (1944). The solutions are then made up to 10 ml with water.

The extinctions at 239 $m\mu$ of the first two series of solutions are measured, using the third series as blanks. Then the pH of each solution in the first two series is accurately determined. Curves are plotted to show extinction-pH relationships. These curves show the pH range over which the extinction of the interfering substances is constant, and they show the constants K and k (defined below).

Extraction The tissues are thoroughly homogenized (Potter & Elvehjem, 1936). The homogenate is transferred quantitatively into a narrow glass stoppered measuring cylinder. Absolute ethanol (2 vol.) is added and the mixture is shaken and allowed to stand for 15 min. Water ($\frac{1}{3}$ vol.) is added and the volume of the resulting solution noted. The mixture is then filtered through a Whatman no. 42 paper. The clear filtrate is saturated with NaCl. One half of it is taken into a separating funnel, acidified with a drop of concentrated HCl, and extracted once with an equal volume of ether.

The aqueous layer is discarded. The ethereal layer is extracted once, in the same separating funnel, with approx 5 ml. of 0.2M-NaOH. The alkaline layer is run off, and most of the ether is removed by a current of N_2 . The solution is then made up to 10 ml with water.

Spectrophotometry Portions of this alkaline solution (5 ml.) are placed in each of two test tubes graduated at 10 ml. The pH values of these two samples are adjusted to the different desired values by the addition of appropriate volumes of phosphate or borate solutions, the volumes are adjusted to 10 ml with water. The extinctions at 239 $m\mu$ are then determined.

Calculation The two solutions submitted to spectrophotometry are designated S_1 and S_2 , and their hydrogen ion

concentrations pH_1 and pH_2 respectively, where $\text{pH}_1 > \text{pH}_2$. The following symbols are also used

- R_1 = Extinction of S_1
- R_2 = Extinction of S_2
- x_1 = Extinction of impurities in S_1
- x_2 = Extinction of impurities in S_2
- K = Ratio of extinctions at 239 $m\mu$ of the barbiturate at pH_1 and pH_2 . This is a constant, the value of which is found by reference to curves obtained from the preliminary experiments (e.g. Fig. 2)
- k = Ratio of extinctions at 239 $m\mu$ of the barbiturate at pH_1 and pH_10 . This is also a constant and its value is obtained from the same curves
- M = Mol. wt. of the barbiturate (Table 1)
- ϵ_{max} = Molecular extinction at 239 $m\mu$ of the barbiturate at pH_10 (Table 1)
- W = Weight of tissue (g)

First, the extinction of the impurities in S_1 and S_2 must be calculated. If, as is usually the case, $x_1 = x_2$, it is clear that

$$K = R_1 - x_1 / R_2 - x_1 \quad (1)$$

$$\text{Therefore } x_1 = KR_2 - R_1 / K - 1 \quad (2)$$

The concentration of barbiturate in S_1 and S_2 is

$$(\lambda R_1 - x_1) \frac{10^3 M}{\epsilon_{\text{max}}} \mu\text{g/ml} = \left[\lambda R_1 - \frac{KR_2 - R_1}{K - 1} \right] \frac{10^3 M}{\epsilon_{\text{max}}} \mu\text{g/ml} \quad (3)$$

In the procedure suggested the tissue barbiturate is diluted 1 in 40, therefore the concentration of barbiturate in the tissue is

$$\left[\lambda R_1 - \frac{KR_2 - R_1}{K - 1} \right] \frac{4 \times 10^4 M}{W \epsilon_{\text{max}}} \mu\text{g/g} \quad (4)$$

This formula can be simplified if it can be shown that the extinction due to the impurities does not change significantly when the pH is varied even more, i.e. from 10 to 6.5 or lower. At pH 10 barbiturates absorb maximally, below pH 6.5 they cease to absorb altogether. Therefore (3) reduces to

$$(R_1 - R_2) \frac{10^3 M}{\epsilon_{\text{max}}} \mu\text{g/ml}, \quad (5)$$

$$\text{and (4) to } (R_1 - R_2) \frac{4 \times 10^4 M}{W \epsilon_{\text{max}}} \mu\text{g/g} \quad (6)$$

If the extinction due to the impurities does not remain absolutely constant between pH_1 and pH_2 , but shows small, determinable, variations, it is possible to correct the error introduced thereby, as follows.

If $x_2 = nx_1$ (1) may be written

$$K = R_1 - x_1 / R_2 - nx_1, \quad (7)$$

$$\text{and } x_1 = KR_2 - R_1 / nK - 1 \quad (8)$$

Therefore, (4) becomes

$$\left[\lambda R_1 - \frac{KR_2 - R_1}{nK - 1} \right] \frac{4 \times 10^4 M}{W \epsilon_{\text{max}}} \mu\text{g/g} \quad (9)$$

and (6) becomes

$$(R_1 - R_2/n) \frac{4 \times 10^4 M}{W \epsilon_{\text{max}}} \mu\text{g/g} \quad (10)$$

Table 1 Molecular weights and molecular extinctions of barbital and pentobarbital

Compound	Mol. wt. (M)	Concentration		Observed extinction at 239 $m\mu$ and pH 10	Molecular extinction coefficient ϵ_{max}
		($\mu\text{g/ml}$)	(mm/l)		
Barbital	184.2	10	0.054 ₃	0.570	10,500
Pentobarbital	228.3	10	0.044 ₁	0.441	10,000

Table 2 *Experimental data on solutions examined*(Buffers added S_1 , borate (0.5M), S_2 , phosphate (1.0M))

Tissue extracted wet wt (300-500 mg)	S_1			S_2			K	λ
	Vol of buffer added ml	pH ₁	Extinction observed (E , 1 cm)	Vol of buffer added ml	pH ₂	Extinction observed (E , 1 cm)		
Rat liver	2.3	10.0	0.184	1.06	8.0	0.180	—	—
Rat liver	2.3	10.0	0.116	1.06	8.0	0.119	—	—
Rat kidney	2.3	10.0	0.126	3.97	6.4	0.128	—	—
Rat heart muscle	2.3	10.0	0.104	3.97	6.4	0.102	—	—
Barbital	2.3	10.0	0.572	1.06	8.0	0.377	1.52	1
Pentobarbital	2.3	10.0	0.440	1.06	8.0	0.258	1.71	1

RESULTS

Known amounts of barbital and pentobarbital were added to pieces of rat tissue, re-extracted and estimated according to the method described. Table 2 shows experimental data and Table 3 the results of recovery experiments. It will be seen that recoveries were satisfactorily constant and close to 100%.

Table 3 *Recovery of barbital and pentobarbital added to various tissues*

Tissue (wet wt 300-500 mg)	Drug added	Wt added (μ g)	Recovery (%)
Rat liver	Barbital	200	95
	Barbital	100	90
	Pentobarbital	200	95
	Pentobarbital	100	94
Rat kidney	Barbital	200	91
	Pentobarbital	200	84
Rat-heart muscle	Barbital	200	92
	Pentobarbital	200	99

DISCUSSION

Published procedures for the estimation of very small amounts of barbiturates are based either on the cobalt colour reaction or on ultraviolet absorption spectrophotometry.

The cobalt colour reaction (Koppanyi, Murphy & Krop, 1933; Koppanyi, Dille, Murphy & Krop, 1934) depends upon the formation of a reddish colour when barbiturates in chloroform solution are treated with a cobalt salt in the presence of isopropylamine or some other weakly alkaline reagent. The test has been repeatedly criticized for its lack of specificity (Riley, Krause, Steadman, Hunter & Hodge, 1940) and for its relative insensitivity. It was originally claimed that the reaction was sensitive to barbital in concentrations of 10 μ g/ml of the chloroform solution used for colorimetry, but Cohen (1946) found that the range of concentrations susceptible to accurate estimation was 1-10 mg/ml. The author

has found that the colorimetric estimation of concentrations smaller than 0.1 mg/ml is difficult and inaccurate. Hellman, Shettles & Stran (1943) stress the fact that the pink colour obtained is transient and difficult to read.

Ultraviolet spectrophotometry has formed the basis of a number of earlier methods for the quantitative determination of micro amounts of barbiturates. The technique of Hellman *et al* (1943) is limited to thiobarbiturates. Dorfman & Goldbaum (1947) reported experiments with barbiturates and referred to a spectrophotometric method (Jailer & Goldbaum, 1946; Goldbaum & Hidalgo, 1949), but no detailed description of their procedure, other than that applicable to pentothal, is available at the time of writing.

Very recently, and unknown to the author until after the completion of the experimental work, Walker *et al* (1948) have described a spectrophotometric method which is based on considerations similar to those underlying the method described in this paper. These workers, after measuring the extinction in aqueous NaOH solution at pH 10, due to barbiturate and to interfering substances, abolish the extinction due to barbiturate by acidification with a micro drop of sulphuric acid, and again measure the extinction. They find that the extinction due to interfering substances extracted from blood does not alter appreciably between pH 10 and a strongly acid reaction, and they are thus able to calculate barbiturate concentration from the simple difference in the extinctions measured. Their formula is, therefore, essentially the same as equation (5) above. They suggest that this method might also prove applicable to tissues other than blood. The present author has not been able to confirm this suggestion in the case of liver extracts, indeed he has observed that the extinctions of such extracts, obtained and treated in a manner closely similar to that of Walker *et al* (1948), show variations of up to 20%. Only over a relatively narrow range of pH have the extinctions been found constant to within the experimental error. Another difficulty

referred to by Walker *et al* (1948) is that not infrequently acidification with strong acid brings about clouding of the solution which is thereby rendered useless for further absorptiometry. This has not occurred in our procedure. Moreover, the recoveries obtained by the method of Walker *et al* (1948) are low, the average being 70 %.

Ultraviolet spectrophotometry permits the estimation of concentrations of barbiturates as low as 1-2 $\mu\text{g/ml}$ in pure solution. Since the sensitivity of a method based on ultraviolet spectrophotometry is limited by the ratio of the extinction by barbiturate to the extinction by interfering matter, attempts have been made to obtain the drugs from tissue extracts in pure solution. Earlier procedures employed charcoal for clearing the extracts (Delmonico, 1939, 1940, Levvy, 1940, Anderson & Essex, 1942), but charcoal adsorbs significant amounts of barbiturate (Hellman *et al* 1943). Hellman and co-workers washed ethereal extracts of the drug from blood with 0.5M-sodium bicarbonate, but according to Walker *et al* (1948) this again causes the loss of some of the drug. Purification of the drugs by chromatography on alumina columns was described by Raventos (1946), and their isolation by sublimation was used by Cohen (1946). Various micro sublimation procedures were tried here, but it was not found possible to ensure purification and quantitative recovery simultaneously.

The present method makes a high degree of purification unnecessary, since it permits accurate estimation of barbiturate concentrations in the presence of unknown impurities. In particular, it has the following advantages.

(1) Barbiturates are highly soluble in 50 % ethanol, whether the reaction is neutral, acidic or basic. Ethanol extraction appears to promote full recovery of the drugs without increasing the amount of impurities extracted.

(2) Absorption spectrophotometry is affected neither by traces of ether left in solution, nor by the variations in the concentrations of the buffering agents, provided use is made of corresponding blank solutions.

(3) The method has been found to be applicable to blood, naturally without preliminary homogenization, and with the following alterations in the procedure for protein precipitation. To 1 vol of whole blood in a boiling tube is added one fifth vol of molar potassium dihydrogen phosphate which brings the pH to between 4.5 and 5.0. The tube is heated on a boiling water bath for 3 min. After cooling, 2 vol absolute ethanol and 1 vol water are added, the mixture shaken and filtered and the procedure is then continued as before.

SUMMARY

1. A procedure based on ultraviolet spectrophotometry is described for the quantitative determination of very small amounts of barbiturates in tissues and blood.

2. Complete elimination of contaminants is not attempted. The difficulty due to the simultaneous extraction of barbiturate and impurities absorbing in the same region of the ultraviolet is overcome by the use of differential spectrophotometry, depending on extinction-pH relationships.

3. The procedure is rapid, accurate and sensitive and appears to offer some advantages over methods previously described.

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Nicotinamide Biosynthesis by Intestinal Bacteria as Influenced by Methyltryptophans

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In recent papers (Ellinger & Abdel Kader, 1947, 1948, 1949b) it has been shown that *Bacterium coli* (*Escherichia coli*) was able to synthesize nicotinamide from ornithine, but not from tryptophan, and that mixed cultures from faeces or caecum contents could synthesize nicotinamide also from tryptophan. It was suggested that in the conversion of tryptophan to nicotinamide, ornithine was formed as intermediate by a cleavage of the tryptophan molecule. It was hoped that the utilization by the intestinal flora of methyltryptophans with the methyl group in various positions, i.e. in the alanine side chain, in the pyrrole and in the benzene nucleus, might throw some light on this theory. This was not so for reasons to be discussed later, but the experiments provided other results which are to be presented in this paper.

METHODS

The experiments were carried out *in vitro* with mixed cultures from rat caecum contents, consisting mainly of coliform bacteria, staphylococci, *Streptococcus faecalis*, plenty of an unidentified coccus growing in very small colonies and pure cultures of *Bact. coli* 4c (Type I faecal, Ministry of Health, 1939). The technique used was mainly that described before (Ellinger & Abdel Kader, 1949b). Growth was measured by assessing opacity with the Brown scale.

The following compounds were tested: DL ornithine, DL tryptophan, DL 2-methyl-, DL-4-methyl-, DL 5-methyl-, and DL 7-methyl tryptophans,* and L- α -methylamino β (3-indolyl) propionic acid (abrin). The 2-, 4-, 5- and 7-methyl tryptophans were kindly supplied by Dr H. N. Rydon who described their preparation and properties (Rydon, 1948). Abrin was prepared from the seeds of *Abrus praecatorius* by a method similar to that of Hoshino (1935). The seeds were cracked and the husks removed, the cotyledons were then ground to a fine powder, dried *in vacuo* over P_2O_5 and extracted with ether to remove as much as possible of a yellow oil which interferes with the precipitation of the abrin. The powder was filtered off and extracted to exhaustion with methanol in a shaking machine at room temperature (5-6 hr). The powder was filtered off and washed with methanol. Filtrate and washings were evaporated to dryness at reduced pressure. When the dry residue was washed with a little cold water the abrin separated as a white powder which was purified by further washings with cold water on the centrifuge. It was then recrystallized several times from hot water, m.p. 292° (decomp.).

Media of ammonium lactate (Fildes, 1938) containing one of the compounds mentioned in 2 mM concentration were incubated with suspensions of mixed cultures of caecum contents of rats. In another series the same media containing

ornithine or tryptophan alone, or ornithine plus either tryptophan or one of the various methyltryptophans, were incubated with a pure culture of *Bact. coli* 4c. In a third series ammonium lactate containing ornithine and one of the five methyltryptophans in concentrations from 0.01 to 2 mM was incubated with *Bact. coli* 4c for 72 hr. In all three series growth and nicotinamide formation were estimated as before (Ellinger & Abdel Kader, 1949b).

The effect of the methyltryptophans on acid production by *Lactobacillus arabinosus* had to be tested, since it was the basis for the assay of nicotinamide. For this purpose the Barton-Wright (1944) media containing one of the methyltryptophans in 2 mM concentration, the concentration present in the assay, were inoculated with a suspension of *Lb. arabinosus* and the acid formed was estimated after incubation for 72 hr at 37°.

In order to obtain information about the effect of the different methyltryptophans on the growth of the various intestinal bacteria, agar plates were inoculated with equal amounts of the 24 hr cultures using standardized Pasteur pipettes and incubated for 48 hr. Separate counts were made for the most frequent bacteria. They were compared with similarly made plates from cultures in pure ammonium lactate.

RESULTS

In pure cultures of *Bact. coli* 4c growth was very little affected by any of the compounds tested (Table 1). Nicotinamide formation from ammonium lactate was slightly inhibited by tryptophan and increased in the usual way by ornithine (Ellinger & Abdel Kader, 1949b). The nicotinamide formation from ornithine was slightly inhibited (by 6%) by tryptophan and by abrin (by 14%) and completely inhibited by 2-, 4-, 5- and 7-methyltryptophans in 2 mM concentration. In smaller concentrations (Table 2) growth was very little affected, and, if so, slightly increased, only once a slight inhibition (by 5%) was observed with one concentration of the 7-methyl derivative, this was within the limits of error of the method. Nicotinamide formation was inhibited to different degrees, rising with the concentrations of 2-, 4-, 5- and 7-methyltryptophans, and hardly inhibited by abrin.

In the experiments (Table 3) using mixed cultures from the caecum content, growth was not markedly affected by any of the compounds tested. The viable counts from 24 hr cultures showed an undiminished growth of coliforms. Nicotinamide production was stimulated by ornithine and tryptophan and by

* The numbers 2, 4, 5 and 7 refer to the positions in the indole nucleus, the usual convention being followed.

Table 1 *Effect of ornithine, tryptophan and ornithine plus tryptophan or one of five methyltryptophans on growth and nicotinamide formation by Bacterium coli 4c in ammonium lactate medium*

Compound tested (2 mM)	Growth (% of control)	Nicotinamide formed	
		(μg /ml medium)	(% of ornithine control)
None	100	10	29
DL-Ornithine	100	35	100
DL Tryptophan	100	8	23
DL Ornithine + DL tryptophan	100	33	94
DL Ornithine + DL 2 methyltryptophan	100	0	0
DL Ornithine + DL 4 methyltryptophan	100	0	0
DL Ornithine + DL 5 methyltryptophan	100	0	0
DL Ornithine + DL 7 methyltryptophan	100	0	0
DL Ornithine + L abrin	100	30	86

Cell content of inoculum about 6×10^8 /ml, of cultures after 48 hr growth, about 380×10^6 /ml

Table 2 *Effect of various concentrations of 2-, 4-, 5- and 7 methyltryptophans and of abrin, on growth and nicotinamide formation by Bacterium coli 4c in ornithine ammonium lactate medium*

Concentrations of methyltryptophans (mM)	2 Methyltryptophan			4 Methyltryptophan		
	Growth (% of control)	Nicotinamide formed		Growth (% of control)	Nicotinamide formed	
		(μg /ml)	(% of control)		(μg /ml)	(% of control)
0	100	29	100	100	26	100
0.01	125	20	69	100	18	69
0.03	125	20	69	100	15	58
0.1	125	15	52	100	14	54
0.3	125	6	21	100	6	23
1.0	100	25	9	100	5	19
2.0	100	0	0	100	0	0

Concen- trations of methyl tryptophans (mM)	5 Methyltryptophan			7-Methyltryptophan			Abrin		
	Growth (% of control)	Nicotinamide formed		Growth (% of control)	Nicotinamide formed		Growth (% of control)	Nicotinamide formed	
		(μg /ml)	(% of control)		(μg /ml)	(% of control)		(μg /ml)	(% of control)
0	100	29	100	100	26	100	100	29	100
0.01	125	28	96	100	14	54	100	29	100
0.03	125	27	93	100	13	50	100	29	100
0.1	125	23	79	100	6	23	150	28	96
0.3	125	14	48	100	3.7	14	150	27	93
1.0	100	6	21	95	2.5	10	100	26	89
2.0	100	0	0	100	0	0	100	25	86

Inoculum for 2- and 5 methyltryptophans and for abrin, 62×10^8 cells, and for 4- and 7-methyltryptophans 61×10^8 cells, growth of control after 72 hr, 76×10^6 cells/ml

Another similar experiment gave similar results

Table 3 *Effect of ornithine, tryptophan and five methyltryptophans on growth and nicotinamide formation by mixed cultures from rat caecum contents in ammonium lactate medium*

Compound tested (2 mM)	Growth (% of control)		Nicotinamide formed			
			(m μg /ml medium)		(% of control)	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
None	100	100	10	6	100	100
DL Ornithine	100	64	22	56	220	933
DL Tryptophan	100	100	18	27	184	450
DL 2 Methyltryptophan	100	100	6	0	60	0
DL 4-Methyltryptophan	100	100	6	0	60	0
DL-5 Methyltryptophan	155	100	6	0	60	0
DL 7 Methyltryptophan	155	100	8	0	80	0
L Abrin	100	100	19	29	186	483

Four more experiments showed complete inhibition in one case and partial inhibition in three cases by 2, 4, 5- and 7 methyltryptophans

abrin to the same extent as by tryptophan. In Exp 1, Table 3, it was inhibited to a considerable but varying degree, and in Exp 2 completely, by the 2-, 4-, 5 and 7 methyltryptophans in concentrations of 2 mM

Table 4 *Effect of the five methyltryptophans on the acid production by Lactobacillus arabinosus in Barton Wright medium*

Compound tested (2 mM)	Acid produced/ml medium	
	(ml 0.1 N NaOH)	(% of control)
Control	0.672	100
DL 2 Methyltryptophan	0.680	101
DL 4 Methyltryptophan	0.684	102
DL 5 Methyltryptophan	0.684	102
DL 7 Methyltryptophan	0.254	37
L Abrin	0.692	103

Growth of *Lb arabinosus* in the Barton-Wright medium was inhibited by 7-methyltryptophan and unaffected by the others (Table 4). This allows the use of the bacterium for quantitative assay for all but the 7-methyl derivatives. Since the inhibition of *Lb arabinosus* by this compound was incomplete, it could be concluded that the 7-methyltryptophan also interfered with the nicotinamide synthesis by *Bact coli*.

The viable counts of the mixed cultures from the caecum contents did not show any marked diminution of the main representatives of the intestinal flora by any of the methyltryptophans in 2 mM concentration.

DISCUSSION

Inhibition of growth by the various methyltryptophans with methyl groups in the indole nucleus has been observed for *Bacterium typhosum*, for which tryptophan is an essential nutrient, by Fildes & Rydon (1947). *Bact coli* does not need tryptophan for growth, and, as would be expected, the methyltryptophans in concentrations up to 2 mM did not interfere markedly with the growth of *Bact coli*, although Anderson (1945) observed complete inhibition of growth of a certain strain by 5-methyltryptophan (*Bz*-3-methyltryptophan) in concentrations of 18 μ M. The difference might be due to the strain, the nature of which is not described by Anderson. The non-utilization of the 2-, 4-, 5- and 7-methyltryptophans by *Bact coli* made it impossible to draw any conclusions on the chemical mechanism of the tryptophan nicotinamide conversion. The similarity of the action of abrin and that of tryptophan is paralleled by the findings of Gordon & Jackson (1935) that growing rats fed on a tryptophan-free diet can utilize abrin and suggests that in both cases a demethylation of the α -N atom takes place. The 2- and 5-methyltryptophans seemed to have an action antagonistic to tryptophan in Gordon & Jackson's experiments. The fact that, in the experiments with mixed cultures, nicotinamide synthesis was entirely inhibited in only two cases

and partly in the four others might be due to the fact that, in the mixed culture, part of the methyltryptophans might have been used up by non coliform bacteria.

In lower concentrations small differences in inhibition of nicotinamide synthesis by the various methyl derivatives were observed, but they were too small to justify the drawing of any conclusion on the relation between degree of inhibitory action and molecular structure, as discussed by Fildes & Rydon (1947) for *Bact typhosum*. The inhibition of the nicotinamide formation by the 2-, 4-, 5- and 7-methyltryptophans, in the light of the theory of the action of metabolite antagonists as formulated by Fildes (1940), suggests that tryptophan is normally involved in this process. The mode of this action is obscure and needs further investigation. In a recent paper Heidelberg, Gullberg, Morgan & Lepkowsky (1948) have shown that after administration of DL tryptophan, labelled with 14 C in the β position, to rabbits, dogs and rats the urine contained labelled kynurenine and kynurenic acid. The nicotinamide methochloride isolated from the urine of all three species was not radioactive. It was surprising that this compound was isolated from the urine of rabbits since these animals are known not to synthesize this compound in normal circumstances (for literature, see Ellinger & Abdel Kader, 1949a). These findings, combined with the findings of this paper, suggest the possibility that the nicotinamide saving action of tryptophan is not due to a direct conversion, but to a stimulating effect of tryptophan on the biosynthesis mechanism. If this supposition should prove to be correct it would entirely change our conception of the nicotinamide saving action of tryptophan, and attribute to tryptophan a catalytic coenzyme like action on the enzyme system involved in the synthesis of nicotinamide.

The difference in the action of tryptophan on the nicotinamide synthesis by mixed cultures of intestinal bacteria on one hand and that by pure cultures of *Bact coli* on the other (Ellinger & Abdel Kader, 1949b) could be explained in the following way: pure *Bact coli* produces sufficient tryptophan to maintain the optimum rate of nicotinamide synthesis, while in mixed cultures non coliform organisms consume so much tryptophan that extra tryptophan is needed for a maximum formation of nicotinamide. The inhibition of the growth of *Lb arabinosus* by 7-methyltryptophan alone of all methyltryptophans examined is also obscure. The tryptophan-like action of abrin might be due to the greater instability of this compound which is probably demethylated easily to tryptophan.

SUMMARY

1. The effect of 2-, 4-, 5- and 7-methyltryptophans and of abrin on growth and nicotinamide formation by pure cultures of *Bacterium coli* (*Escherichia coli*)

and mixed cultures of rat caecum contents and on the acid production by *Lactobacillus arabinosus* has been studied

2 Growth of *Bact coli* or of mixed cultures was not markedly affected by any one of the methyl-tryptophans in the concentrations used, that of *Lb arabinosus* was considerably inhibited by the 7-methyl compound

3 Nicotinamide synthesis from ornithine or ammonium lactate by *Bact coli* was completely inhibited by 2 mM and correspondingly less by lower concentrations of 2, 4, 5 and 7-methyltryptophans,

but not by abrin Nicotinamide synthesis by mixed bacteria from the rat caecum contents was either completely or partly inhibited by the former at 2 mM and not affected by the latter compound These findings suggest that tryptophan is involved in the bacterial biosynthesis of nicotinamide

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The Linkage of Glutamic Acid in Protein Molecules

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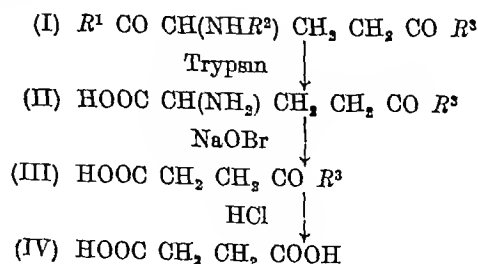
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The experiments presented in this paper were carried out to answer the question as to whether the γ -carboxyl groups of glutamic acid are involved in the formation of bonds in protein molecules The possible existence of such bonds in proteins is suggested by the fact that γ linked glutamic acid residues have been found in glutathione, in the capsular substance of *Bacillus anthracis* (Bovarnick, 1942, Hanby & Rydon, 1946) and in folic acid (Boothe, Mowat, Hutchings, Angier, Waller, Stokstad, Semb, Gazzola & Subbarow, 1948) Different modes of combination of the γ carboxyl groups of glutamyl residues in proteins can be considered (1) formation of a peptide bond with the terminal amino group of a peptide chain, (2) ester linkages with hydroxyl groups of hydroxy amino acids and (3) thio-ester linkages with cysteine molecules of a peptide side chain (Chibnall, 1942) Since the γ substituted glutamyl residues could give rise to branching of the main peptide chain, the problem of γ substitution is of great importance

In order to test proteins for the presence of γ -substituted glutamyl residues we subjected them

to the following series of procedures (a) partial digestion with trypsin, (b) oxidation, (c) extraction of the acidified solution with ether (ether extract 1), (d) total hydrolysis, (e) extraction of the acid hydrolysate with ether (ether extract 2) and (f) determination of succinic acid in the last ether extract Succinic acid found in ether extract 2 was considered as originating from the γ glutamyl residues The above mentioned method is based on the fact that the γ -peptide bond of glutathione is more resistant to trypsin than are the normal α -peptide bonds (Grassmann, Dyckerhoff & Eibeler, 1930, Kendall, Mason & McKenzie, 1930) It was expected, therefore, that at least a part of the α - and γ -substituted glutamyl residues (formula I) would be transformed into γ -glutamyl peptides (formula II) by the action of trypsin



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The reactions (b)–(e), by which the γ -glutamyl peptide (II) is oxidized to the succinyl peptide (III) and hydrolyzed to succinic acid (IV), have been used by Quastel, Stewart & Tunnicliffe (1923) and by Kendall *et al* (1930) in their work on glutathione. Their methods had to be modified because of the small amounts of succinic acid to be expected.

EXPERIMENTAL

Partial hydrolysis with trypsin Casein (Merck) and edestin (Schuehardt) were commercial preparations. Ovalbumin was prepared according to Kekwick & Cannan (1936), serum albumin from ox serum by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$, and haemoglobin from horse blood by the ethanol method (Hoppe Seyler, 1878). Since native ovalbumin and serum albumin are scarcely attacked by trypsin, these proteins were denatured by keeping their aqueous solutions in a boiling water bath for 30 min. We are grateful to Dr H. N. Rydon (Birkbeck College, University of London) for a sample of the capsular substance of *B. anthracis*. Each of the proteins (2 g), dissolved or suspended in water, was mixed with 0.2 g of trypsin (Merck) and 1 ml of toluene. The pH was adjusted to 8.5 by the addition of N-NaOH and the volume brought to 100 ml by adding water. The mixtures were kept at 38° for 24 hr, those containing the albumins for 48 hr. The pH was kept near 8.5 by adding small amounts of N-NaOH from time to time. The rate of hydrolysis was determined in control tests run under the same conditions with 200 mg of protein and 20 mg of trypsin. At 0, 24 and 48 hr, 2 ml of neutralized 30% (w/v) formaldehyde and 0.2 ml of a 1% solution of phenolphthalein were added and the amount of hydrolysis determined by titration with 0.1 N-NaOH . The volumes of NaOH recorded in Table 1 include those added to the protein samples to maintain the pH at 8.5.

Table 1 *Action of trypsin on proteins*

(Protein (200 mg), trypsin (20 mg), extent of hydrolysis determined by formol titration using 0.1 N-NaOH . Figures in brackets show increases in NaOH titres over those at zero time.)

Protein	0.1 N-NaOH used for neutralization		
	0 hr (ml.)	24 hr (ml.)	48 hr (ml.)
Casein	2.8	8.5 (5.7)	10.8 (8.0)
Edestin	0.64	6.5 (5.9)	7.6 (7.0)
Ovalbumin	0.85	4.9 (4.0)	7.2 (6.3)
Serum albumin	2.2	6.5 (4.3)	8.0 (5.8)

The amount of the capsular substance of *B. anthracis* was too small to permit formol titration. All of this substance (50 mg) was treated with 5 mg of trypsin in 5 ml of water for 24 hr.

Oxidation The tryptic hydrolysate from 2 g of protein was mixed at 0° with 240 ml of a cold 1% (v/v) solution of Br_2 in 0.5 N-NaOH . After 5 min the mixture was acidified with glacial acetic acid, and 20 g of KI were immediately added. The volume of the solution was reduced to 40–50 ml by evaporation *in vacuo* and HCl was added until congo red paper gave a blue colour. The solution was extracted continuously with ether for approximately 100 hr (ether extract 1). It was then mixed with 2 vol. of concentrated HCl and refluxed for 4 hr. The hydrolysate was evaporated to a small volume, diluted with water and extracted with ether for 15 hr (ether extract 2).

Determination of succinic acid The ether extracts were evaporated, dissolved in a small volume of water, filtered and evaporated to dryness. To the residues, dissolved in 1 ml of water, an excess of finely powdered $\text{Ba(OH)}_2 \cdot 8\text{H}_2\text{O}$ and 9 ml of ethanol were added. The insoluble Ba succinate was centrifuged, dissolved in dilute HCl, evaporated to dryness and extracted repeatedly with 3–5 ml of ether. The ether extracts were evaporated, dried, weighed and analyzed for their succinic acid content according to Krebs (1937), washed pigeon-breast muscle being used as source of enzyme. The results of the analyses are shown in Table 3.

Control experiments In Exps 1–7 and 10 (Table 4) no trypsin was used. The substrates were oxidized directly by NaOBr . Casein (Exp 1) was dissolved in 100 ml of water by adding the required amount of N-NaOH . Casein hydrolysate (Exp 2) was obtained by boiling 0.5 g of casein with 20 ml of 20% (w/v) HCl for 4 hr, concentrating and neutralizing the aqueous solution. In Exps 8 and 9 the substrates were treated with one tenth of their weight of trypsin and kept at 38° for 24 hr. The amino acid mixture (Table 4, Exp 10) contained 100 mg of each of the following: glycine, alanine, serine, cystine, valine, leucine, isoleucine, aspartic acid, phenylalanine, tyrosine, arginine and histidine, together with 50 mg of lysine, 50 mg of tryptophan and 100 mg of glucose. In all these experiments (see Table 4) the oxidation, extraction and hydrolysis with HCl were carried out as described in the preceding sections. In another control experiment glutamic acid (100 mg) was boiled with 20 ml of 23% (w/v) HCl containing 1 g of KI and approximately 0.1 g of I_2 . After 4 hr the solution was extracted continuously with ether and the extract examined in the usual manner. No succinic acid was found.

Determination of glutamine in the tryptic hydrolysate Each of the hydrolysates (0.5 ml equivalent to 10 mg of protein) was brought to pH 3.5 by the addition of 0.1 N-HCl and kept

Table 2 *Determination of glutamine and of pyrrolidone carboxylic acid in the tryptic hydrolysate*

Protein	Consumption of 0.04 N-HCl		Found in 2 g of protein		
	Before heating	After heating	Pyrrolidone carboxylic acid (mg)	Glutamine (mg)	Total glutamic acid (mg)
	(ml.)	(ml.)			
Casein	0.025	0.060	26	41	440*
Edestin	0.020	0.062	21	49	410*
Ovalbumin	0.106	0.190	109	99	320*
Serum albumin	0.011	0.035	11	28	340†
Haemoglobin	0.010	0.032	10	26	170‡

* Chibnall (1946) † Shemin (1945) ‡ Foster (1945)

in a boiling water bath for 2.5 hr. The amount of NH_3 present before and after boiling was determined according to Conway (1933), 0.04N HCl being used for the titration of the NH_3 absorbed by the boric acid (Table 2). Asparagine is not split noticeably under these conditions (Chibnall & Westall, 1932).

RESULTS

Table 1 shows that 35–40% of the peptide bonds of the proteins examined were split by the action of trypsin under the conditions of our experiments. Since γ -glutamyl bonds are slowly hydrolyzed by water at 38°, the duration of the digestion was reduced by employing relatively large amounts of trypsin. Table 4 (Exp. 9) shows that a considerable part of the glutathione resisted hydrolysis under the same conditions, but that another part was split as shown by the yield of succinic acid in ether extract 1.

The oxidizing agents used by Quastel *et al.* (1923) and by Kendall *et al.* (1930) were hydrogen peroxide, hypobromite or chloramine T. When the methods of these authors were applied to proteins, large amounts of succinic acid were found in the ether extract 2 (Haurowitz & Vardar, 1944). Later investigations showed that the destruction of hydrogen peroxide and of hypobromite is not complete in these methods, so that a further oxidation occurs during the hydrolysis with hydrochloric acid (Schwerin, Kara & Tanasoglu, unpublished results).

Much lower results were obtained with chloramine T, which can easily be removed from the reaction mixture, but since the action of chloramine T requires temperatures of 40°, and since γ -glutamyl bonds are unstable at these temperatures in acid solutions, we preferred to use hypobromite at 0°. Goldschmidt & Strauss (1929) removed the excess of hypobromite by adding the calculated amount of hydrogen peroxide. It is hardly possible, however, to avoid a small excess of either peroxide or hypobromite. We therefore destroyed the peroxide by an excess of potassium iodide added in slightly acid solution. The bulk of the iodine formed was removed by the first ether extraction, so that it could not act as an oxidant during the subsequent acid hydrolysis.

Table 3 shows that 2 g. of protein on oxidation furnished 8–29 mg. of succinic acid before hydrolysis with hydrochloric acid (ether extract 1), and only 0.8–1.7 mg. of succinic acid after hydrolysis with acid (ether extract 2). The succinic acid found in ether extract 1 originates from the oxidation of glutamic acid and glutamine, both present in the tryptic hydrolysates of proteins. Only the succinic acid in ether extract 2 can arise from γ -glutamyl peptides of the formula II.

In view of the small quantities of succinic acid found in the ether extracts 2, it was very important to remove all of the succinic acid which had been

Table 3 *Determination of succinic acid in partially digested and oxidized proteins before and after hydrolysis with hydrochloric acid*

Protein	Wt (g)	Ether extract 1 (before HCl hydrolysis)			Ether extract 2 (after HCl hydrolysis)		
		Wt (mg)	Succinic acid in		Wt (mg)	Succinic acid in	
			2 g of protein (mg)	10 ⁵ g of protein (mol)		2 g of protein (mg)	10 ⁵ g of protein (mol)
Casein	2.0	88	29	12	15	1.5	0.64
Edestin	2.0	121	24	10	18	1.1	0.47
Ovalbumin	2.0	92	14	6	30	0.8	0.34
Serum albumin	2.0	138	23	10	12	1.1	0.47
Haemoglobin	2.0	120	8	3	39	1.7	1.4
Capsular substance of <i>B anthracis</i>	0.05	3	0.3	5	1.6	0.48	8.1

Table 4 *Determination of succinic acid in control experiments*

Exp. no.	Substrate	Wt (g.)	Hypo bromite solution added (ml.)	Ether extract 1		Ether extract 2	
				Total wt (mg.)	Succinic acid (mg.)	Total wt (mg.)	Succinic acid (mg.)
1	Casein	5.0	200	5	0.4	6	0.6
2	Casein hydrolysate	0.5	190	85	37	0	0
3	Glutamic acid	0.09	24	63	45	0	0
4	Proline	0.05	5	3	1.0	0.4	0
5	Hydroxyproline	0.1	10	0.4	0	0	0
6	Methionine	0.1	27	—	0.12	0	0
7	Pyrrolidone carboxylic acid	0.2	20	9.7	4.6	0.6	0.07
8	Glutamine	0.1	26	28	18	0.97	0.01
9	Glutathione	0.128	30	9.4	4.7	11.2	8.0
10	Amino acid mixture	1.4	140	35.7	2.0	—	—

formed before hydrolysis. For this reason the extraction with ether was continued for several days, until the extracts were free even of traces of succinic acid. Under these conditions no succinic acid passed into ether extract 2 after the oxidation and hydrolysis of glutamic acid, proline, hydroxyproline, methionine and of the amino acid mixture examined (Table 4, Exps 2-6). On the other hand, glutathione gave rise to large amounts of succinic acid in extract 2.

Glutamine and pyrrolidonecarboxylic acid gave traces of succinic acid in ether extract 2 (Table 4, Exps 7 and 8). It was necessary, therefore, to determine both these substances in the tryptic hydrolysates. Since pyrrolidonecarboxylic acid is formed from glutamine, its amount is approximately equivalent to the quantity of ammonia found before heating at pH 3.5, while the amount of glutamine is equivalent to the ammonia formed during the heating. According to Table 2 the amount of pyrrolidonecarboxylic acid varies from 10 to 109 mg, that of glutamine from 26 to 99 mg/2 g of protein digested. The amounts of succinic acid to be expected from these quantities of glutamine and of pyrrolidonecarboxylic acid, according to Table 4, are so small that they would not noticeably affect the values of succinic acid passing into the ether extract 2 (Table 3).

DISCUSSION

The amount of succinic acid found after hydrolysis of the oxidized peptides is very small (Table 3). If molecular weights of 100,000, 309,000, 44,000, 68,000 and 68,000 are assumed for casein, edestin, ovalbumin, serum albumin and haemoglobin respectively, the number of γ glutamyl bonds per protein molecule would be 0.64, 1.45, 0.15, 0.32 and 0.95. These figures, however, have to be considered as minimum values. The actual number of γ -glutamyl bonds is certainly higher for the following two reasons: (a) a

considerable part of the γ -glutamyl bonds is split during the treatment with trypsin at 37°, as shown by the experiment on glutathione (Table 4), (b) less than one molecule of succinic acid is formed, when glutamic acid or glutathione are oxidized (Table 4, Exps 3 and 9). We are thus unable to calculate the real number of γ glutamyl bonds per protein molecule, and we have to content ourselves with the result that such bonds are present and that their number in the proteins examined is very low. The assumptions of Chibnall (1942) and of Haurowitz & Vardar (1944), that some of the γ carboxyl groups of glutamic acid are linked to other groups in protein molecules, are supported by these results. Much larger quantities of succinic acid were found in the ether extracts 2 of the capsular substance of *B. anthracis*. According to Hanby & Rydon (1946) this substance has a molecular weight of 50,000 or more, and is built up exclusively of chains of 50-100 α glutamyl residues interlinked by γ glutamyl bonds. Our results agree with this view. The problem of chain branching is not solved by our results for the γ linked glutamyl residues can be bound according to the formula $R-NH-CH(COOH)-CH_2-CH_2-CO-R$, without any ramification of the peptide chains.

SUMMARY

1. Casein, edestin, ovalbumin, serum albumin, haemoglobin and the capsular substance of *Bacillus anthracis* were partially digested with trypsin, and the resulting peptide mixture was tested for the presence of γ glutamyl peptides by oxidizing it with sodium hypobromite. Succinic acid was removed by ether extraction and the succinyl peptides, insoluble in ether, were hydrolyzed by hydrochloric acid.

2. The amounts of succinic acid obtained by this procedure indicate the presence of a small number of γ -glutamyl bonds in the proteins examined, and of a much higher number of such bonds in the capsular substance of *B. anthracis*.

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Displacement Chromatography on Synthetic Ion-exchange Resins

2 THE SEPARATION OF ORGANIC ACIDS AND ACIDIC AMINO ACIDS BY THE USE OF ANION-EXCHANGE RESINS

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The application of the principle of displacement chromatography to the separation of bases and amino acids on columns packed with the cation-exchange resin Zeo Karb 215 has been described in Part 1 of this communication (Partridge & Westall, 1949). It is expected that this method will prove to be of value in the isolation of bases and amino acids from biological extracts of various kinds, and that it will also facilitate the fractionation of protein hydrolysates on a rather larger scale than has been possible hitherto. Since all the amino acids commonly found in proteins are adsorbed by Zeo-Karb 215 and other cation exchangers of a similar type, the use of these resins constitutes a means of carrying out the first fractionation of a protein hydrolysate into a series of groups of amino-acids of similar affinity for the cation exchanger.

The data given in Part 1 show that although aspartic acid should be separable from a protein hydrolysate in a pure condition, glutamic acid will appear in a mixed fraction containing serine and threonine, thus further means are required to separate glutamic acid from the two hydroxyamino-acids. Since several excellent methods have been described for the separation of glutamic and aspartic acids from each other (Drake, 1947, Consden, Gordon & Martin, 1948) and also from other less acidic amino acids (Freudenberg, Walch & Molter, 1942, Cleaver, Hardy & Cassidy, 1945, Tiselius, Drake & Hagdahl, 1947) by the use of synthetic resinous anion exchangers, the application of a chromatographic technique based on one of the commercially obtainable anion exchange resins obviously presents itself. However, the methods cited have been designed, for the most part, for use on a smaller scale than is envisaged here, and for that reason it was considered worth while to reinvestigate a number of anion exchangers by methods conforming to the general pattern of those described in Part 1.

EXPERIMENTAL

Materials

Samples of three commercial resins have been examined. These were Wofatit M, Amberlite IR 4 (Resinous Products

and Chemical Company, Philadelphia), De Acidite B (Pormutit Company, London). We are indebted to the Director of the Chemical Research Laboratory, Teddington, for the gift of the sample of Wofatit M, supplies of which are at present difficult to obtain.

Methods

The apparatus used and the methods adopted for the determination of the adsorption capacity of the resins and the width of the boundaries are generally as described in Part 1. It should, however, be pointed out that the behaviour of the anion exchangers is markedly different from that of the highly acidic base exchange resins in several important respects. These differences in behaviour are due especially to the fact that exchange reactions taking place on the anion exchangers are much slower than those observed on resins containing $-\text{SO}_3\text{H}$ groups. In addition, commercial resins of the condensed polyamine type contain weakly acidic groups, which give the resin an amphoteric character in neutral or weakly alkaline solutions. This latter property causes considerable difficulty in regenerating the resin, particularly as it is desirable to prepare the resin base in a substantially salt-free condition.

Various methods of regeneration have been used to suit particular experimental conditions, and details of the procedures adopted appear in the relevant sections below.

The determination of titration curves. The experimental procedure was identical for the three resins investigated. The sample of resin was ground in the dry condition in a hammer mill and graded between wire sieves (80–100 mesh/in). The powder was allowed to stand overnight in 0.1N HCl and was washed free from fine particles by means of an upward flow of water. It was then packed into a tall glass filtration tube and treated three times alternately with 0.1N HCl and N-NaOH, the final washing from N-NaOH being carried out with a large volume of distilled water until the specific conductivity of the effluent solution was less than 10 gommhos. The powdered resin was then dried in air at room temperature and a sample taken for the determination of moisture content by drying to constant weight over P_2O_5 *in vacuo* at 80°. Twenty to thirty samples of the air dried resin, each about 0.6 g, were then weighed into 50 ml flasks, 20 ml of 0.5N NaCl were added to each flask and the volume made up to 40 ml by the addition of various amounts of HCl or NaOH. The flasks were then tightly stoppered and allowed to stand with occasional shaking for 48 hr, when the supernatant solution was withdrawn from each flask for titration and for the determination of pH (glass electrode).

RESULTS

The effect of pH on the retention of anions The anion-exchange resins at present available commercially are rather complex in structure, and usually contain a wide range of reactive groups which vary considerably in their strength as bases. The following functional groups are commonly present in such resins: —NH_2 (aromatic or aliphatic), =NH (aromatic or aliphatic), $\equiv\text{N}$ (aliphatic) (cf Myers, 1942, Davies, 1948). In addition, weakly acidic phenolic hydroxyl groups may be present.

The complexity of the ionizing system in such resins may be illustrated by the example of one commercial anion exchanger which is said to be prepared by the co condensation of ethylenediamine with phenol and formaldehyde, cross links being formed by the reaction of the condensed product with ethylene dichloride. Such a resin would be expected to contain —NH_2 (aliphatic), =NH (aliphatic), $\equiv\text{N}$ (aliphatic) and —OH (aromatic), and its titration curve would, therefore, show the presence of a whole series of ionizing groups over a considerable pH range.

The titration curves of three commercial resins are given in Fig 1. The determinations were carried out by the method already given, and it should be observed that the curves represent the adsorption of Cl^- or Na^+ from solutions containing a relatively high concentration (0.25M) of sodium chloride. In the curves, the amount in m-equiv of Cl^- or Na^+ adsorbed/g (dry wt) of resin is plotted against the pH of the supernatant solution. It will be seen that in each case the adsorption of Cl^- increases steadily with decreasing pH from pH 9 to 1, and that at pH 9–10 and above there is a net adsorption of Na^+ . Thus the resins (in different degrees) have weakly acidic properties in addition to their main character as bases.

With each sample of resin there is a point in the region of pH 9–10 at which the well washed resin retains neither free acid nor free base and thus behaves as an amphoteric substance at its isoelectric point, but it should be noted that at this point the adsorption capacity of the resin is not necessarily zero, since both cations and anions may be removed from the solution in equal amounts. Indeed in the case of a resin containing phenolic hydroxyl as the base-reacting radical, the adsorption of cations at pH 9–10 may be considerable, and, if the resin has an 'isoelectric point' in this region, it must be expected to retain both anions and cations in significant amounts when equilibrated with weakly alkaline solutions containing salts. This expectation is confirmed by experience, since, if the resins are brought to their free base condition by buffering to pH 9–10 with borate or carbonate-bicarbonate

mixtures, and are subsequently washed with distilled water, the removal of salt (or free alkali produced by hydrolysis of the salts of weak acids) from the resin proves to be an extremely slow process, considerable quantities of electrolyte are washed from the resin before the conductivity of the effluent solutions approaches that of the distilled water used for washing.

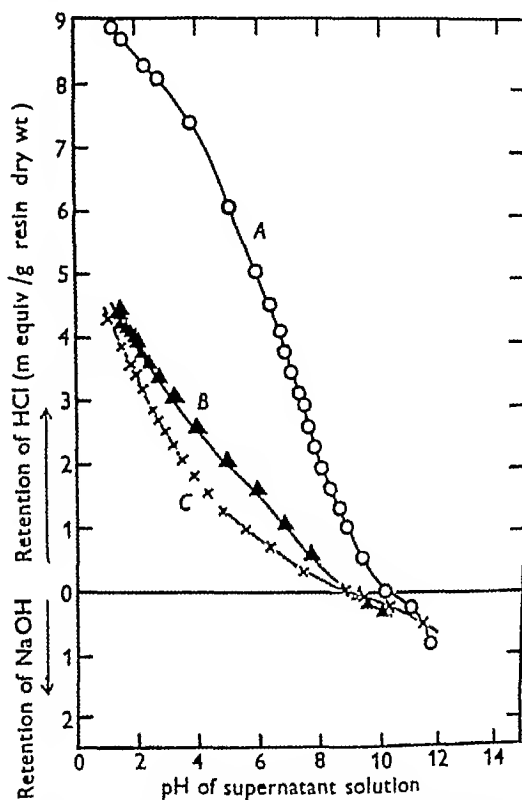


Fig 1 Titration of three commercial anion exchange resins in the presence of 0.25M NaCl: curve A, Amberlite IR4, curve B, Wofatit M, curve C, De Acidite B.

This is an important consideration wherever large quantities of resin are to be used, and, in fact, the amount of distilled water required to wash a large column free from electrolytes after it has been regenerated with alkali or alkaline buffers may be prohibitive. For the practical purposes of this work, it has, therefore, been considered preferable to choose a cycle of operations which avoids the necessity of bringing the resin into equilibrium with alkaline solution, and, as will appear later, a further advantage in limiting the adsorption regeneration cycle to pH 1–7 lies in the fact that, over this range, expansion and contraction of the resin is reduced. However, some of the small-scale laboratory experiments (such as the determination of adsorption isotherms) call for a fully regenerated resin, and these were carried out with resin samples that had been regenerated with dilute sodium hydroxide solution.

Titration curves for a sample of Amberlite IR4 have recently been given by Kunin & Myers (1947), who carried out the determinations both in the presence and the absence of neutral salt. These curves show clearly that the free-base form of the resin may be brought, by exhaustive washing, into equilibrium with distilled water at a pH approaching 7, although the resin (which contains primary aliphatic amino groups) is a fairly strong base. This is to be expected since the resin is insoluble, and charge effects tend to prevent the release of free hydroxyl ions into the aqueous phase. On addition of a neutral salt, however, the pH of the solution rises, presumably because soluble cations become available, and the supernatant solution acquires a concentration of hydroxyl ions sufficient to bring about an equilibrium of essentially the same kind as that occurring when a soluble base is dissolved in water containing electrolytes.

The width of the boundaries In Part I an expression was derived by which the width (λ_0) of a boundary formed by a solute on a column could be calculated from a knowledge of the shape of the solute front in the effluent

$$\text{Width of the boundary in cm } (\lambda_0) = \left(\frac{V_{90} - V_0}{V_e} \right) L$$

The notation used is that given in Part I. The results of a number of such determinations on the cation-exchange resin Zeo-Karb 215 are shown in Part I, Fig 6 (p 425), which shows the effect of the particle size of the resin and the rate of progression of the boundary down the column on the width of a boundary due to 0.042N-NH₃.

Fig 2 shows the widths of boundaries due to 0.0495M glutamic acid on various anion-exchange resins. The experimental procedure adopted was that described in Part I (p 425). Curve A (Fig 2) gives the widths of the boundaries on a column of 9.0 mm diameter \times 103 mm height packed with De Acidite B (80–100 mesh/in). The De Acidite B, after treating alternately with N-NaOH and 0.1N-HCl, was regenerated with N-NaOH solution and washed with distilled water to a low conductivity. Curve B was obtained with Amberlite IR4 (80–100 mesh/in) which was regenerated by equilibration with 0.0005N-HCl after treating alternately with 0.1N-HCl and water. Curve C was obtained with Wofatit M (80–100 mesh/in) which was regenerated by the method used for the sample of De Acidite. Curve D refers to a column packed with Amberlite IR4 that had been treated alternately with 0.1N-HCl and water and regenerated with phosphate buffer of pH 7.0 followed by thorough washing with distilled water to low conductivity.

It will be observed that, with the anion-exchange resins, the effect of the rate of flow in the column is very much more marked than is the case with Zeo-

Karb 215, and that the boundaries (with comparable rates of flow) are very much wider in all cases. Indeed it is clear that, with all the samples, the rate of progression of the boundary down the column must be limited to less than 2 cm/hr in order to obtain separations of the same sharpness as those taking place on Zeo-Karb 215 at a comparable rate of 15 cm/hr.

The adsorption of a weak acid such as glutamic acid on the anion exchangers is, therefore, a very slow process as compared with the replacement of H⁺ by cations on strongly acidic resins such as Zeo-Karb 215, and, since parallel experiments with aspartic acid and lactic acid gave similar results, it

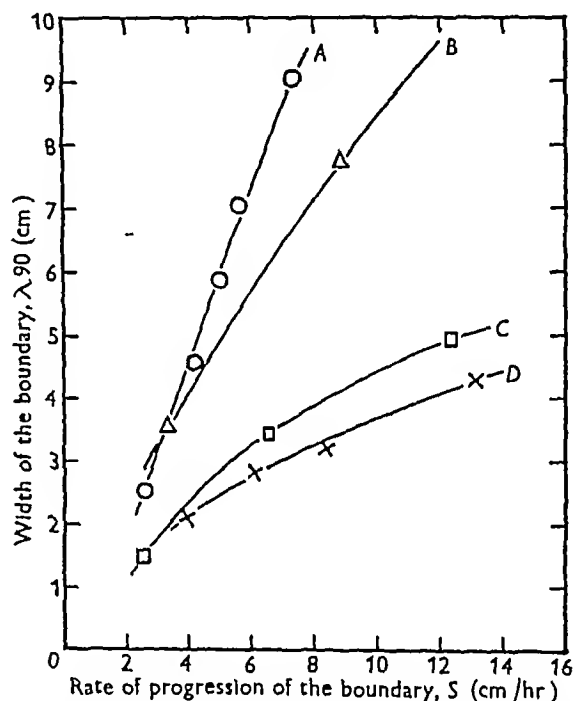


Fig 2 Width of boundaries formed by glutamic acid (0.05M) on various anion-exchange resins (80–100 mesh/in): curve A, De Acidite B; curve B, Amberlite IR4 (regenerated with 0.0005N HCl); curve C, Wofatit M; curve D, Amberlite IR4 (regenerated with phosphate buffer of pH 7).

may be assumed that the slow rate of reaction applies to organic acids as a whole. Since the boundary widths found for 80–100 mesh/in resin were so large, it was not considered useful to examine the properties of resin samples of larger particle size, and the finer grade of resin was used throughout the remainder of the investigation, in separation experiments the rate of progression of the boundaries down the column was adjusted to 3–4 cm/hr, since slower rates than this were not considered to be practical except under special circumstances.

The data given in Fig 2 provide a rational basis for the choice of a suitable resin for the purpose of fractionation experiments. Curve D shows that the

boundaries on the column of Amberlite (regenerated to pH 7) are considerably narrower than those given by any of the other resin samples. However, the adsorption capacity of the resin should also be taken into account in considering the performance of a column since it is clear that although the boundary given by Amberlite IR4 (regenerated to pH 7) is 2.75 cm at a rate of progression of 6 cm/hr, and that given by the same resin regenerated with 0.0005N-HCl is 5.75 cm, in the former case the capacity of the resin for anions is about twice that in the latter. Thus for a given quantity of solute the band on the column is approximately one-half the height when the column is regenerated to pH 7, and consequently the proportion of pure solute recoverable should be approximately the same in both cases. However it must be borne in mind that regeneration to pH 7 would allow the use of a shorter column.

For the purpose of comparing the performance of resins, we may therefore compare the values of $\lambda_{90} \times \epsilon$ for a given solute at a given rate of progression of the boundary. This comparison is set out in Table 1 for boundaries given by glutamic acid, and, from these data, it is clear that the performances of all the anion exchange resins on this basis do not greatly differ.

The suitability of a resin for a given separation must remain a matter to be decided in relation to particular circumstances regarding both the type of separation and the apparatus available. Resins of lower adsorptive capacity suffer the disadvantage that longer columns are required for the same throughput, but in the present work it was considered preferable to use long columns packed with De Acidite B since the chemical and mechanical stability of this resin made it convenient to handle in the finely powdered condition.

The data for the cation-exchange resin Zeo Karb 215 are included in Table 1 to serve as a basis for comparison. The superiority of the cation exchanger at high rates of flow is most striking, and is in conformity with our experience in fractionation experiments on the two kinds of resin. Displacement chromatography on commercial anion-exchange resins was successful only if carried out slowly and on long columns packed with finely ground material.

The retention of organic acids on De Acidite B
Fig 3 shows a number of 'retention isotherms' which were determined on a sample of De-Acidite B (1.60 g dry wt) that had been treated several times alter-

nately with 0.1N-HCl and N-NaOH, and regenerated with N-NaOH solution. The values of ϵ were calculated from the retention volumes (V_e) given by a column 58 mm in height and 9.0 mm in diameter. Details of the experimental procedure for the determination of ϵ are given in Part 1, p. 421.

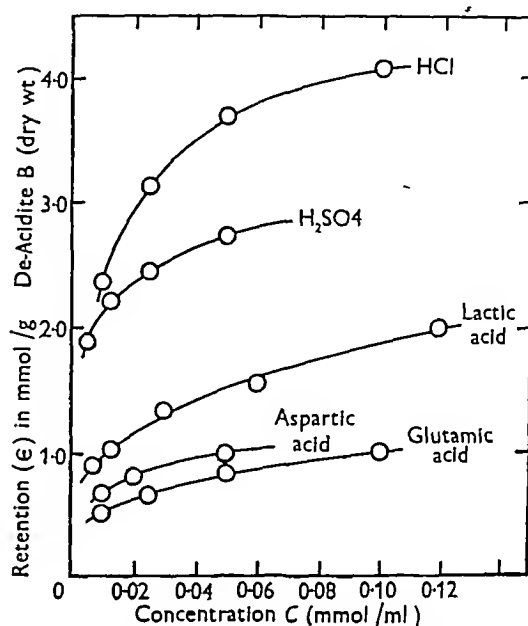


Fig 3 The retention of a number of acids on De Acidite B (80-100 mesh/in). The experiments were carried out with a column 9 mm in diameter containing 1.6 g resin.

The adsorption of the neutral amino acids, serine, glycine and alanine, on De-Acidite B was very small and for practical purposes may be neglected. The two acidic amino acids, aspartic acid and glutamic acid, were well adsorbed, and, as would be expected from its lower isoelectric point, the value of ϵ (mmol of solute adsorbed/g resin dry wt) for aspartic acid was rather higher than that for glutamic acid. A feature of the group of curves given in Fig 3 is that the adsorption capacity of the resin for the strong mineral acids is three to four times that for aspartic and glutamic acids. This was to be expected from the titration curve of the resin (Fig 1) which demonstrates the presence of a range of basic groups of varying strengths. Thus hydrochloric acid may be retained by weakly basic groups in the resin which remain un-ionized in the presence of the acidic amino acids.

Table 1 Comparison of $\lambda_{90} \times \epsilon$ for glutamic acid boundaries on various resins

Resin	ϵ (mmol/g)	S (cm/hr)	$\lambda_{90} \times \epsilon$	S (cm/hr)	$\lambda_{90} \times \epsilon$
De Acidite B (fully regenerated)	0.85	5	5.1	10	10.8
Amberlite IR4 (regenerated to 0.0005N HCl)	0.80	5	4.0	10	6.8
Wofatit M (fully regenerated)	2.15	5	6.2	10	9.5
Amberlite IR4 (regenerated to pH 7)	2.10	5	5.0	10	7.8
Zeo Karb 215 (acid form)	2.20	5	2.2	10	2.6

The curves in Fig 3 may be used in the same way as those in Fig 3 (Part 1) for the calculation of the concentration at which one acid is displaced by another. Inspection of the curves shows that in order to displace aspartic acid as an 0.05M solution by means of a mineral acid, a concentration of about 0.25N-HCl or 0.40N-H₂SO₄ would be required. The high concentrations of mineral acids required must be regarded as a disadvantage in chromatographic experiments, since they increase the risk of chemical degradation of the resin with consequent release of nitrogen containing compounds, and also tend to increase the swelling suffered by the resin. For this reason lactic acid was used as the displacement developer in some of the displacement experiments, but this was of doubtful advantage since in one experiment bacteria were observed to be growing on the top part of the column in the presence of lactic acid.

The separation of aspartic acid, glutamic acid and serine It has already been pointed out that the separation of serine from glutamic acid is of importance in the fractionation of the amino acids of protein hydrolysates, since these two amino-acids form a mixed band when displaced from Zeo-Karb 215 by means of dilute ammonia solution. The separation of serine from glutamic acid should proceed readily on De-Acidite B since serine is adsorbed to a very small extent on this resin. Aspartic acid and glutamic acid may be separated fairly readily on cation-exchange resins when serine is present, but it was nevertheless considered useful to investigate the behaviour of the two acidic amino acids on an anion-exchange resin.

Fig 4a gives the results of a separation experiment in which serine (2.3 mmol), glutamic acid (2.3 mmol) and aspartic acid (2.3 mmol) were dissolved in water (75 ml) and applied to a column of De-Acidite.

The column was 11.5 mm in diameter and contained 10.66 g air dried De-Acidite (dry wt 6.97 g). The resin had been treated alternately with N-NaOH and 0.1N-HCl and regenerated with N-NaOH followed by washing with distilled water until the specific conductivity of the effluent fell below 10 gemmhos. After washing, the resin occupied a height in the filtration tube of 15.6 cm. The displacement developer used was 0.101M-lactic acid (concentration as determined by titration with alkali), and this solution was applied at a rate of about 75 ml/hr. Since approximately 130 ml lactic acid solution were required to saturate the column, this rate of flow corresponded with a rate of progression of the lactic acid boundary down the column of 9 cm/hr. The effluent was collected in 10 ml fractions, and a qualitative analysis of each fraction was obtained by setting up one dimensional filter-paper chromatograms (Consden, Gordon & Martin, 1944).

The results are given in the form of block diagrams in Fig 4a. The figure shows that serine was well separated from the two acidic amino acids and was retarded by the column to a small extent only. A partial separation of aspartic from glutamic acid was obtained, but the separation was not sufficiently complete to have much practical value. From an analysis of the block diagram, the width of the boundary (λ_{90}) on the column between aspartic acid and glutamic acid was about 6.8 cm, and, since the rate of progression of the front was 9 cm/hr, the width of the aspartic-glutamic boundary was not greater than would be expected for a single solute front travelling down the column at the same rate (Fig 2, curve A).

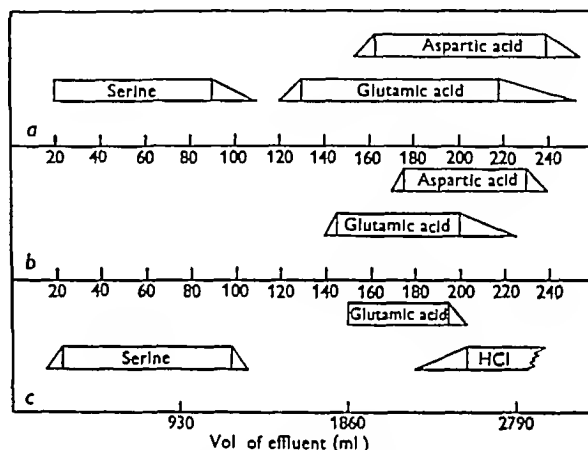


Fig 4 (a) Separation of serine, glutamic acid and aspartic acid by displacement with 0.10M lactic acid ($S=9$ cm/hr) (b) Separation of aspartic acid and glutamic acid by displacement with 0.10M lactic acid ($S=1.8$ cm/hr) (c) Separation of serine and glutamic acid by displacement with 0.51N-HCl

This indicated that an improvement in the separation should be obtainable at low rates of progression of the boundaries. To test this a second experiment was set up using the same column. A mixture of aspartic acid (0.306 g) and glutamic acid (0.339 g) in water (80 ml) was applied to the column and a solution of the same concentration (0.101M) of lactic acid was used as displacement developer. The application of the developer was adjusted to give a rate of progression of the lactic acid boundary of 1.8 cm/hr. The result of the experiment is shown in Fig 4b, inspection of which shows that the overlap between the aspartic and glutamic acid bands was considerably reduced. However, calculation of the width of the aspartic-glutamic acid boundary shows this to be about 3.0 cm, and thus, at the low rate of flow, the boundary width was greater than the width of a single-solute boundary (1.60 cm) given by extrapolation of curve A (Fig 2) to $S=1.8$ cm/hr.

The results of these two experiments, taken together with those from confirmatory experiments

on the same lines, indicate that the separation of glutamic acid and aspartic acid can only be successful when carried out very slowly, and for this reason it was considered that for practical purposes the method would be of doubtful value, particularly as the two acidic amino-acids are readily separated on cation exchange columns. The use of the anion-exchange resins, however, provides the simplest method for separating serine from glutamic acid.

Procedure for the regeneration of De-Acidite The difficulty of washing the commercial anion exchange resins free from electrolytes after they have been regenerated with strong alkalis has been emphasized already. For experiments with small columns the consumption of distilled water used in the washing procedure after regeneration with sodium hydroxide solution is unimportant, but for larger columns the quantity of distilled water required becomes prohibitive. Nevertheless, complete removal of alkali is necessary for all separations involving weak acids, since its presence raises the pH of the solution in contact with the resin, and thus tends to inhibit the adsorption of the acid. Effects of this sort were met with in experiments with glutamic acid, which failed to form sharp fronts on columns that had been insufficiently washed after regeneration with sodium hydroxide.

Various alkalis and buffer solutions were investigated for use in the regeneration of the resins. The use of sodium carbonate or bicarbonate was undesirable since the evolution of carbon dioxide during the regeneration process necessitated the repacking of the column before each experiment. Attempts were made to use various buffer solutions of pH 8–10, but these also were rather unsatisfactory since the removal (by washing with water) of the excess of electrolyte over this pH range proved to be a slow process. It therefore appeared to be desirable to avoid the use of buffer solutions more alkaline than pH 7, and regeneration with sodium acetate solution was finally adopted as a routine procedure. Details of the procedure are illustrated in the following example.

A column of De Acidite (125 g dry wt, 80–100 mesh/in) contained in a filtration tube 3.62 cm in diameter occupied a height of 26 cm, and had been saturated with 0.25N-HCl. A solution of sodium acetate (0.25N, 6 l) was then passed slowly through the column until the effluent solution reached pH 6 (time 4 hr). The column was then washed with distilled water (10 l) until the conductivity of the washings fell to 25 gemmhos. At this point the wash water had an acid reaction (pH 4.5), but was free from chloride.

Separation of serine from glutamic acid on a larger scale The column used contained 125 g (dry wt) De-Acidite B (80–100 mesh/in) and was 3.62 cm in diameter and 26 cm in height. The resin had been

regenerated with sodium acetate solution as described in the previous section. A solution of L glutamic acid (80 g) and DL serine (150 g) in water (1 l) was allowed to flow through the column, and the effluent solution was collected in 93 ml samples by means of an automatic fraction collector (Brimley & Snow, 1949). The time required for this operation was 4.3 hr. After washing with a small quantity of water (25 ml) the reservoir was filled with 0.51N-HCl (2 l), and the solution allowed to flow through the column at a rate of 140 ml/hr. The conductivity of the effluent was measured at intervals throughout the run, and the experiment was stopped when the rise in conductivity showed that the hydrochloric acid boundary had broken through (time 10 hr, vol of HCl solution 1.4 l).

The pH of each 93 ml fraction was measured and a small quantity of solution was taken from each for chromatographic analysis by the filter-paper method. The chromatogram obtained is illustrated in Fig. 4c, which shows that serine was well separated from glutamic acid. The serine solution emerged from the column at pH 5.4, while the glutamic acid band emerged at pH 3.35, except for the last three fractions which were mixed with hydrochloric acid.

Since hydrochloric acid at a concentration of 0.51N displaces L glutamic acid at a concentration higher than its solubility in water (Fig. 3) glutamic acid crystals appeared in the effluent solution soon after the fractions were taken. The serine solution (fractions 2–14 inclusive) contained a small concentration of acetic acid derived from the sodium acetate used to regenerate the column. The solution was concentrated under reduced pressure, treated with a small amount of charcoal and finally evaporated to dryness under vacuum at room temperature (yield 1.5 g colourless crystals, N, found, 13.2%, theory, 13.3%). The glutamic acid solution was collected in two parts, fractions 20–24 inclusive (free from hydrochloric acid) and fractions 25–27 (containing a fairly high concentration of hydrochloric acid). Both portions were evaporated to dryness after treatment with a little charcoal. Fractions 20–24 yielded glutamic acid as a colourless crystalline product (4.73 g, N, found, 9.9%, theory, 9.5%). The residue from fractions 25–27 was weighed as glutamic acid hydrochloride (pale yellow crystals, 2.2 g). Total yield calculated as glutamic acid 6.5 g, recovery 81%.

The separation of glutamic acid from aspartic acid by development with acetic acid Drake (1947) described an interesting analytical method for the separation of glutamic acid from aspartic acid which could be carried out with a few mg of material. A column of Amberlite IR4B was used, and the separation was carried out by development with acetic acid, which is adsorbed on anion exchange resins approximately to the same extent as glutamic,

but less strongly than aspartic acid. With the concentration of acetic acid used (0.05%) the development results in the formation of an acetic acid band lying between and overlapping the two amino-acid bands, and, since the complete elution of aspartic acid requires a large volume of acetic acid, Drake introduced a second developer (N-HCl) in order to displace the aspartic acid at a suitable concentration.

In view of the rather poor results obtained in the separation of glutamic acid from aspartic acid by the direct application of displacement development, it was considered worth while to investigate an adaptation of the method of Drake to preparative separations involving relatively large quantities of amino-acids. Details of a typical separation experiment are given below.

The column contained De-Acidite B (80–100 mesh, dry wt. 125 g) and was 26 cm high \times 3.62 cm in diameter. The resin had previously been regenerated with sodium acetate solution and washed thoroughly with distilled water. A solution (400 ml) containing glutamic acid (4.0 g) and aspartic acid (1.0 g) was passed through the column (time, 3 hr) and development was carried out with 0.102N-acetic acid (1.3 l, time, 8 hr). The reservoir containing the acetic acid was then replaced by one containing 0.25N-HCl, and this solution (2.5 l) was allowed to flow into the column for a further 8 hr.

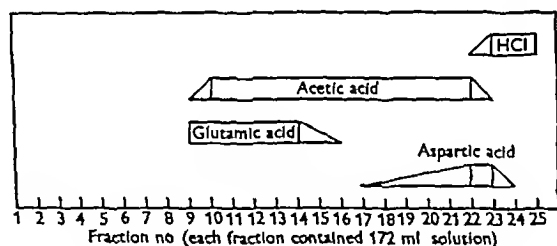


Fig 5 Separation of glutamic acid from aspartic acid on De-Acidite B. The column was developed with 0.1N-acetic acid followed by 0.25N HCl.

The effluent solution was collected in 172 ml fractions, and a one-dimensional paper chromatogram was made from samples taken from each fraction. The chromatogram is illustrated in Fig 5 which shows complete separation between the aspartic and glutamic acids. The positions of the acetic and hydrochloric acid bands (found by titration) are also indicated in the diagram. It will be observed that the acetic acid front coincided with that due to glutamic acid, while the aspartic acid band was to some extent overlapped by the hydrochloric acid band. Fractions 9–16 inclusive (Fig 5) were mixed and the solution concentrated under reduced pressure and evaporated to dryness *in vacuo* over sodium hydroxide. The product (glutamic acid) was a colourless crystalline solid (yield 3.27 g, N,

found, 9.8%, theory, 9.5%). The yield of glutamic acid represented a recovery of 82%. Thus, although separation was complete, there was a loss of 0.73 g of glutamic acid. In the experience of the authors, losses of this order are usual with both aspartic and glutamic acid where commercial anion exchangers are employed, and it is considered that the poor yields may in part be due to secondary adsorption of a difficultly reversible character. Chemical degradation of the amino-acids in contact with the resins may also be a contributory factor.

DISCUSSION

In the work described above, three commercial anion-exchange resins were investigated with particular reference to their suitability for the purpose of displacement chromatography on a preparative scale. The two acidic amino acids, aspartic acid and glutamic acid, were well adsorbed by the resins, and the main object of the work was to establish suitable conditions for the separation of these two substances from each other and from mixtures containing neutral amino acids. The procedures adopted were based upon those developed for the separation of bases and amino acids on cation-exchange resins (Partridge & Westall, 1949).

The titration curves of the commercial anion-exchange resins show that they are markedly poly-functional in behaviour, they contain a range of basic radicals of varying strength and also show a weakly acidic character. When in equilibrium at reactions near neutrality they behave as ampholytes, and, for this reason, regeneration of the resins must be carefully controlled. If strong alkalis are used in the regeneration process, cations are adsorbed by the resins which are difficult to remove completely by subsequent washing. Failure to effect the complete removal of alkali after the regeneration process gives rise to a reduction in the adsorptive capacity of the resin for weak acids, since the residual cations become involved in salt formation with the acids, and this increases the pH of the solution in contact with the resin. For this reason, regeneration with strong alkalis should be avoided when dealing with large columns. De-Acidite B may be regenerated effectively with sodium acetate solution, and with this reagent only small volumes of wash water are required to render the columns free from cations and from anions other than acetate.

The anion exchange resins at present available commercially are relatively weak bases (containing aliphatic and aromatic amino groups as the most basic radicals), and, probably for this reason, the exchange reaction between anions is much slower than that taking place between cations on the strongly acidic resin Zeo-Karb 215. This slowness of reaction is reflected in the broad boundaries shown

by the bands on columns packed with the anion-exchange resins, and measurements of boundary widths at different rates of flow showed that the separation of weak acids by displacement chromatography could only be successful at very slow rates of flow, and by the use of the resins in a finely ground condition

A number of trial separations using mixtures of aspartic and glutamic acids indicated that, while separations of about the expected order of efficiency were in fact obtained, the rate of flow required was very much slower than for the separation of the same pair of amino acids on Zeo-Karb 215. Taking the evidence as a whole, it is considered doubtful if the direct application of the displacement technique to the separation of weakly acidic substances on the anion exchange resins available at present is of practical value

A particularly useful feature of the resins is that the adsorption of neutral amino-acids is very small, and columns packed with the resins may be used as simple adsorptive filters in order to remove aspartic or glutamic acid from mixtures containing the neutral amino-acids. The separation of glutamic acid from serine and threonine is of particular importance in the fractionation of protein hydrolysates since these three amino-acids form a mixed band on Zeo-Karb 215. The separation of serine from glutamic acid was carried out using De-Acidite B and the experiment gave a quantitative recovery of serine, but a loss of about 20 % of the glutamic acid was observed. Losses of this order are a general feature of the adsorption of the acidic amino-acids on the anion exchange resins, and it is considered that the effect is mainly due to the irreversible adsorption of part of the material. However, a further explanation of the loss may lie in the occurrence of oxidative degradation on the surface of the resin particles

Although the direct application of the displacement technique to the separation of aspartic and glutamic acid was unsatisfactory, it still remained possible that a combination of elution and displacement would give satisfactory results as a preparative method. Such a method was developed as a microanalytical technique by Drake (1947) who, by developing a column of Amberlite IR4 with dilute acetic acid, was able to effect a complete separation between the two amino-acids. Acetic acid is adsorbed to roughly the same extent as glutamic acid, but is less strongly adsorbed than aspartic acid, and development with this reagent results in the separation of the two amino acid bands by the interpolation of an acetic acid band between them. An adaptation of this method, using De-Acidite B, gave good results, and a complete separation was obtained using a mixture containing 5 g. of the amino-acids on a column containing 125 g. of the resin

The results of the investigation as a whole show that the anion exchange resins at present available are much less efficient for the purpose of displacement chromatography than are resins of the sulphonated phenol formaldehyde type. This may in large part be due to the polyfunctional character of the anion exchangers and to the rather weakly basic character of the main functional groups. However, the resins may be used with success for the separation of aspartic and glutamic acid from mixtures containing the neutral amino acids

SUMMARY

1 Three commercial anion exchange resins have been investigated for their suitability for displacement chromatography on a preparative scale. These were Amberlite IR4, Wofatit M and De-Acidite B.

2 Titration curves for the three resins are given. These were carried out in the presence of neutral salts and show that the resins have weakly acidic properties in addition to their main function as insoluble bases.

3 The boundary widths given by glutamic acid on columns packed with the anion exchangers are approximately inversely proportional to the adsorptive capacity of the resins. The boundaries given by the anion-exchange resins are much broader at useful rates of flow than those given by the cation exchanger Zeo-Karb 215. This indicates that the exchange reaction is much slower in the former than in the latter case.

4 Some separation was obtained between glutamic acid and aspartic acid on De-Acidite B, but very slow rates of flow were required to give useful results, and (with the anion exchange resins at present commercially available) the technique is of doubtful value for practical purposes.

5 The anion-exchange resins are, however, useful for the separation of glutamic or aspartic acid from neutral amino-acids, particularly serine. In this case, since the resins adsorb neutral amino-acids to a small degree only, separation may be achieved by application of a simple process of adsorptive filtration.

6 Using De-Acidite B, glutamic acid and aspartic acid may be separated on a preparative scale by developing the column with acetic acid, this forms a band lying between and overlapping the bands due to the amino acids.

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Displacement Chromatography on Synthetic Ion-exchange Resins

3 FRACTIONATION OF A PROTEIN HYDROLYSATE

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In the first two parts of this series (Partridge & Westall, 1949, Partridge & Brimley, 1949) the application of displacement chromatography to the separation of simple mixtures of bases, acids and ampholytes has been described, columns packed with ion-exchange resins were used and most of the experiments were concerned with two typical commercial products, the cation exchanger Zeo Karb 215 and the acid-adsorbing resin De-Acidite B. Interest has been particularly directed towards the problem of separating mixtures of amino acids, since these frequently occur in biological extracts of all kinds, and are almost certain to be met with in any attempt to isolate an active biological substance from its source.

An obvious application of the technique lies in the isolation of the optically active amino-acids from the hydrolysis products of proteins. This problem has been of importance to biochemists in the past, and, in view of the advent of the isotope-dilution technique, the successful development of a simple physical method for carrying out such isolations is likely to be of even more importance in the future. It was considered, furthermore, that an attempt to fractionate the products of hydrolysis of a typical animal protein would present the most practical test of the method, and at the same time would yield valuable information concerning the degree of separation and the order of displacement of a wide range of naturally occurring amino acids.

A short account of the fractionation of a protein hydrolysate by displacement development on ion-exchange columns has already been given (Partridge, Westall & Bendall, 1947). In recent years much

work has been published on the isolation of amino-acids from protein hydrolysates by the use of ion-exchange resins in various ways, but since several excellent reviews are available (Martin & Synge, 1945, Cannan, 1946, Tiselius, 1947) no attempt will be made to refer to the original publications in detail. However, most of the methods hitherto employed involve the use of the ion exchange resins as media for elution experiments of the classical type or in simple adsorptive filters. So far as the authors are aware, no systematic attempt to use the displacement technique for this purpose has appeared, although simple mixtures of amino acids have been separated in this way (cf Drake, 1947, Hems, Page & Waller, 1948).

Since commercial egg albumin was readily obtainable, and its amino-acid constitution fairly well known, the product of hydrolysis of this protein with hydrochloric acid was chosen for study. Preliminary experiments showed that the amino-acids tyrosine and phenylalanine behaved irregularly on a column of Zeo-Karb 215, presumably because of the intervention of physical (van der Waals) adsorption by the resin material, which is predominantly aromatic in character. However, since the two aromatic amino-acids are strongly adsorbed by charcoal, and may be eluted from it (Schramm & Primosigh, 1943, Tiselius, 1947) it was possible to remove these two amino-acids from the hydrolysate before proceeding with the separation of the remaining constituents on the cation exchange column. The procedure was found to be particularly advantageous, since in addition to the recovery in a pure condition of tyrosine and phenylalanine, the process

resulted in the complete removal of all coloured material from the hydrolysate before application to the ion-exchange columns

As has already been indicated, theoretical considerations suggest that a fairly large difference in isoelectric point is necessary to secure the separation of two ampholytes on a cation exchange column. A complete separation of all the components of a protein hydrolysate in one passage through the column is therefore not to be expected, the grouping of the isoelectric points suggested rather that the amino-acids would form a series of discrete bands, each containing individuals of closely similar electrical properties. Chromatograms of this type were found in practice, and Table I shows the general arrangement of the bands on a column of Zeo-Karb 215

In displacement chromatography the various bands necessarily overlap each other, and in cutting the fractions it was in most cases necessary to reject the overlapping portions of the bands, or to preserve the mixed solution for working up with a following batch of hydrolysate. Thus the yields obtained from a single fractionation must always show some loss, but high yields should be obtainable from a series of fractionations in which the overlapping portions of the bands are returned to the stock for refractionation

Table I *Amino-acid bands in the order of their displacement from Zeo-Karb 215*

Band	Amino acids
I	Aspartic acid
II	Glutamic acid, serine, threonine
III	Glycine, alanine
IV	Valine, proline
V	Leucines, methionine, cystine
VI	Histidine, unidentified substance
VII	Lysine

Table I shows that, by a single passage through a column of Zeo Karb 215, the amino acids may be separated into seven main fractions, each containing a small number of components. The use of this resin thus offers an excellent means of effecting a first breakdown of the complicated mixture and yields fractions of a kind suitable for the application of further simple chromatographic procedures or further fractionation by other methods such as fractional crystallization, adsorptive filtration, or countercurrent extraction with organic solvents. The distribution and treatment of the bands obtained by first passage through the Zeo-Karb column (using dilute ammonia as displacement developer) may be briefly described as follows

Band I (Table I) needed no further fractionation since it contained aspartic acid only, and this was readily recoverable in a pure crystalline condition by concentrating the solution

Band II contained glutamic acid, serine and

threonine. The formation of a mixed band on Zeo Karb 215 by serine and glutamic acid has already been reported (Part 1, Partridge & Westall, 1949, p 424), and it has been pointed out that the appearance of serine in this position is anomalous since serine is considerably less acidic than glutamic acid. However, serine and threonine are readily separated from glutamic acid on a column of De-Acidite B (Part 2, Partridge & Brimley, 1949, p 518), and this method was used in the present work for the isolation of pure glutamic acid. The remaining components of the band, serine and threonine, were not further separated, but were recovered as a crystalline mixture

Band III contained glycine and alanine and, in the present work, no attempt was made to separate these two amino acids, but they were recovered as a crystalline solid by concentrating the solution

Band IV contained valine and proline. Since these two amino acids occur in egg albumin in rather small proportions, the band containing them was narrow, and the adjacent components overlapped so far into the valine-proline band as to render its isolation impossible with a column of the length used. A cut was, therefore, taken that included Band IV plus the overlapping sections of Band III and Band V, and the solution was passed through a narrow column of the same resin. The proportion of valine and proline in this second solution was much higher than in the original hydrolysate, and thus, on the second narrower column, the valine-proline band was longer and proportionately less overlapped, and a suitable cut could be made which contained valine and proline only. Concentration of this solution yielded the mixture of valine and proline in a crystalline form

Band V contained methionine, cystine and the leucine isomers and, in order to isolate the leucines, advantage was taken of the readiness with which the sulphur containing amino-acids may be oxidized with bromine water. On addition of this reagent to the mixture, both cystine and methionine were converted to oxidation products that were readily separated from the leucines by further application of the chromatographic procedures

Band VI contained histidine and an unidentified basic substance, while Band VII contained lysine. Arginine is more basic than the displacement developer (ammonia) and was not displaced by the latter at the concentration used, but a partial recovery of arginine was obtained from the hydrochloric acid solution used to regenerate the column

The lysine band was well defined, but was overlapped to a considerable extent by ammonia. Lysine did not appear to be present in the chromatogram to the extent estimated from the amino acid analysis of the protein. This was rather unexpected since small-scale experiments with authentic lysine had

previously shown that this amino-acid could be displaced quantitatively by ammonia. However, Hems *et al* (1948) have encountered a similar difficulty, and consider the effect to be due to the presence of the other amino acids in the hydrolysate. As an alternative explanation it may be assumed that there is a small degree of irreversible adsorption of lysine on the resin, and that losses due to this cause are proportionately greater when a small amount of lysine is caused to traverse a long column, than is the case when a similar amount of the amino-acid is handled on a few grams of resin.

It appears from experiments already described (Part 1, p 426) that irreversible adsorption of the basic amino-acids may be due to the effect of phenolic hydroxyl groupings in the resin, which react with bases of the same order of strength as ammonia, but are inert towards weaker bases. With this in mind, experiments are now in progress to investigate the separation of the basic amino acids on a laboratory-prepared resin which contains the sulphonic acid radical, but is free from phenolic OH.

Histidine appeared in the chromatogram as a narrow (but sharp) band in about the proportion that would be expected from the known histidine content of the protein. However, this content is small (approx 2.4%), and, while the recovery of pure histidine would appear to be possible by use of a second narrow column of Zeo-Karb 215, its isolation in this way was not attempted in view of the projected experiments with an improved resin.

EXPERIMENTAL

Hydrolysis of the protein A sample of commercial egg albumin (moisture, 13.8%, ash, 3.4%, 240 g) was hydrolyzed for 40 hr under reflux with 5.5N HCl (2.5 l). The bulk of the HCl was removed by repeated evaporation under reduced pressure, and the residue, after addition of water (1.5 l), was filtered to remove insoluble humin. The filtrate was made up to 2 l and was used as a stock solution.

Treatment with charcoal The charcoal ('Activated Charcoal', British Drug Houses Ltd), 110 g, was first prepared by shaking for 1 hr with 5% (v/v) acetic acid solution (1.5 l) on a mechanical shaker. It was then filtered and thoroughly washed with distilled water. A measured volume of the stock protein hydrolysate solution (650 ml), equivalent to 64 g protein (dry wt), was made up to 4 l with distilled water. The prepared charcoal, which was in the form of a wet filter cake, was transferred to this solution and the mixture shaken gently for 1 hr. The charcoal was then removed, leaving a colourless filtrate, and was thoroughly washed with water (1 l), the washings being added to the bulk of the filtrate.

Recovery of phenylalanine and tyrosine Previous experiments had shown that, under the conditions described above, the charcoal removed tyrosine, phenylalanine, and various coloured substances from the solution. It removed traces only of other amino acids. Elution was carried out by shaking the charcoal for 1 hr with 2 l of 20% (v/v) acetic acid solution containing phenol (5% w/v). The eluted charcoal was removed by filtration, and the supernatant solution

twice shaken out with ether to remove the phenol. The solution, which was almost colourless, was then concentrated to small bulk under reduced pressure and finally evaporated to dryness, leaving a pale straw coloured crystalline residue, which consisted mainly of tyrosine and phenylalanine. The residue was extracted with several successive small quantities of ice cold water, and the insoluble residue (tyrosine) was recrystallized from hot water (yield 1.46 g, or 2.3% on protein dry wt, found N, 7.8, calc for $C_9H_{11}O_3N$, 7.7%).

The water soluble fraction was concentrated under reduced pressure until the solution was slightly viscous, and treated with dry HCl gas at 0°. A heavy crop of crystals of phenylalanine hydrochloride formed and was separated by filtration through a sintered glass funnel. A one dimensional filter-paper strip chromatogram confirmed the purity of the fraction (yield, 1.62 g calculated as free phenylalanine, or 2.5% on protein dry wt, found N, 6.9, calc for $C_9H_{12}O_2NCl$, 7.0%).

Preparation of the cation exchange column The column was in two sections, each 82 cm in height and 3.2 cm in diameter. Both sections were packed with 200 g (dry wt) Zeo-Karb 215 (40-60 mesh/in). The resin was prepared as previously described (Part 1, p 420) and the packed columns were treated alternately with 0.15N-NH₃ and 2N HCl before use in order to consolidate the resin. The column assembly was provided with a conductivity cell arranged for continuous reading, and the solution flowing from the column passed to an automatic fraction collector which was adjusted to collect the solution in 91 ml fractions.

Fractionation on the Zeo-Karb column The solution that remained after treatment with charcoal was made up to 9 l with distilled water and applied to the column at a rate of about 1 l/hr. The solution contained the hydrochlorides of the amino acids and a small excess of HCl, and thus the solution flowing out of the column during this stage of the process was dilute HCl. This was run to waste. The hydrochlorides of the amino acids are adsorbed to a less extent than the free ampholytes, and the dimensions of the column were calculated to be just sufficient to retain the entire amino acid content of the hydrolysate as hydrochloride, thus when displacement with NH₃ was commenced the amino-acid band contracted to less than half its original length, the effluent from the column during this period containing no amino acids. The displacement developer contained 0.148N NH₃ (5.5 l), and the dark brown front due to the NH₃ reached a point 76 cm from the bottom of the column before a sharp rise in the conductivity of the effluent indicated the break through of an amino acid. From this point until the break through of the NH₃ front (determined conductimetrically) the effluent solution was collected in 91 ml fractions. (Time required for displacement with NH₃ solution, 15 hr, rate of progression of the NH₃ boundary, 11 cm/hr).

On standing overnight crystals of aspartic acid appeared in the first few fractions. The pH of each fraction was measured, and small samples were taken from each fraction for the preparation of filter paper chromatograms (Consden, Gordon & Martin, 1944). Fig 1a shows the filter paper chromatogram in which the solvent used was neutral phenol. In this solvent the separation of the more acidic amino acids was good, but the resolution of the more basic amino acids was not sufficient to allow the unambiguous identification of them all. In order to complete the identification of the latter, further chromatograms were carried out using as

solvent butanol acetic acid mixture (Partridge, 1948) and *m* cresol (Consden *et al* 1944) The results of the qualitative analyses carried out in this way are given diagrammatically in Fig 1*b* which also includes specific conductivity and pH readings

It will be observed that the amino acid mixture was distributed in an ordered way over a series of definite bands

(Fig 1*b*) showed that fractions 1-10 inclusive were free from the components of Band II, and these fractions were collected together The combined solution was concentrated to small bulk under reduced pressure and finally evaporated to dryness under vacuum The residue (colourless crystals, 3.92 g) was pure aspartic acid, found N, 10.6, calc for $C_4H_7O_4N$, 10.5% (yield on protein dry wt, 6.1%)

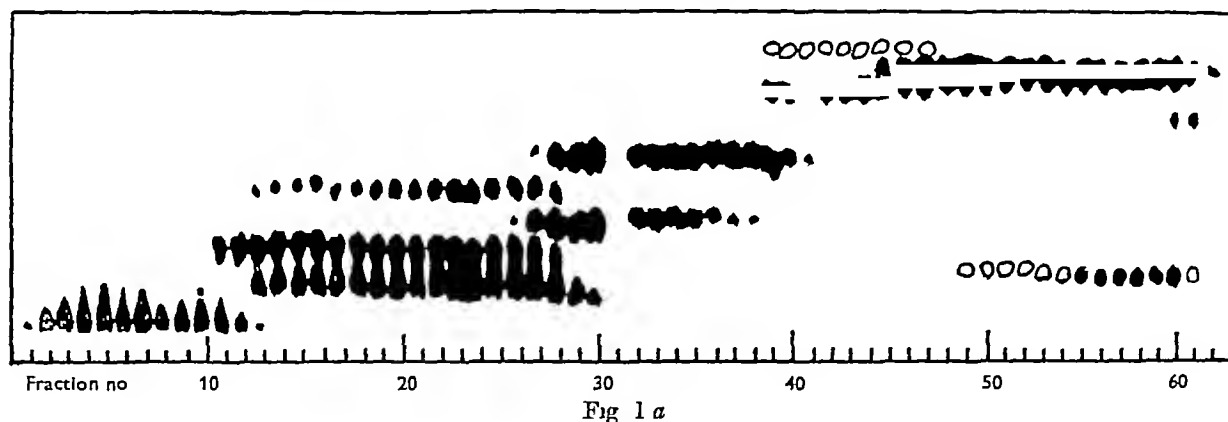
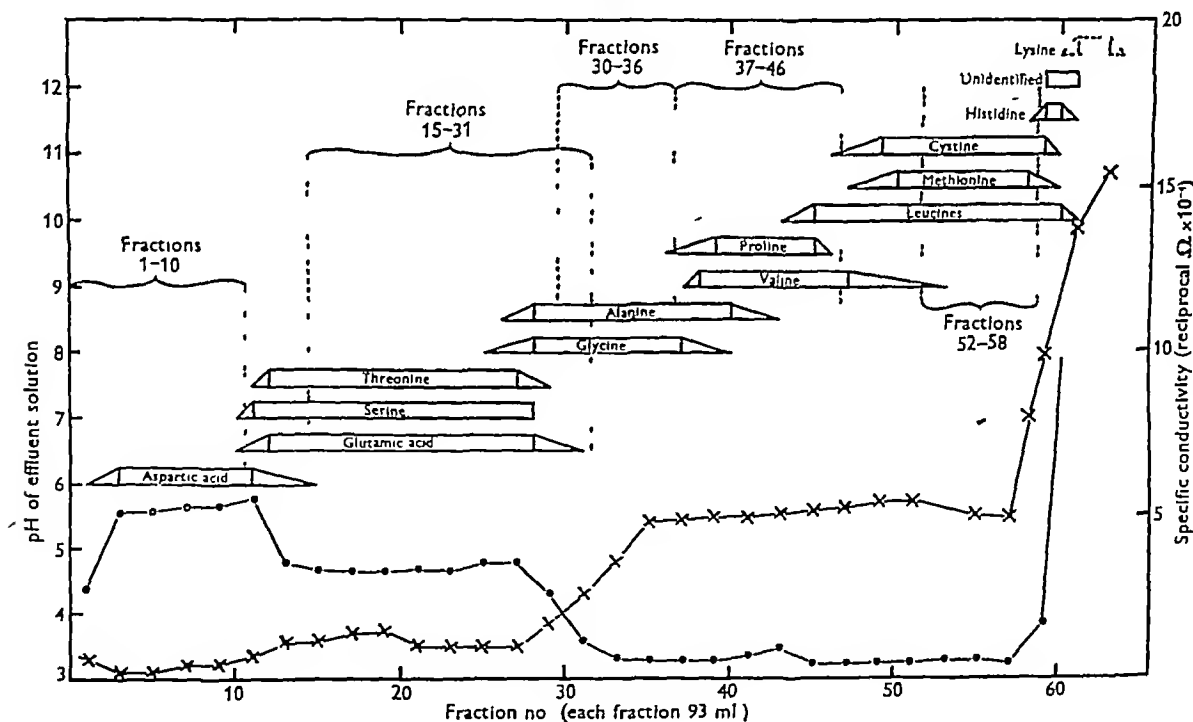
Fig 1*a*Fig 1*b*

Fig 1 Flowing chromatogram obtained by displacing the hydrolysis products of commercial egg albumin from a column of Zeo Karb 215 by means of 0.15N NH_3 . (a) Paper chromatogram carried out on fractions from the flowing chromatogram. The paper chromatogram was carried out in two sections using phenol water as the solvent (Key to amino acids in Fig 1*b*) (b) Diagram showing the position of the cuts made during the collection of the fractions, the amino acids present, the specific conductivity of the effluent $\bullet-\bullet-$ and the pH of the effluent $\times-\times$

The composition of these bands is summarized in Table I which lists the bands in the order of their displacement

Fractionation of bands

Band I aspartic acid This consisted of aspartic acid only, but was overlapped by Band II, containing glutamic acid, serine and threonine The filter paper chromatogram

Band II glutamic acid, serine and threonine The section of Band II that overlapped with Band I (fractions 11-14 inclusive) was rejected, and Band II was collected between fractions 15-31 inclusive The cut was taken in order to exclude aspartic acid, but to include the overlapping section of Band III containing glycine and alanine The fractions were passed, in order, through a column of De Acidite B

which had been regenerated with sodium acetate solution following the procedure given in Part 2 (p 518) The column was 26 cm in height, 3.62 cm in diameter and contained 125 g (dry wt) of resin (80–100 mesh/in) Glutamic acid was retained by the resin, and the neutral amino acids passed through the column with only a small degree of retardation A chromatogram (Fig 2a) carried out on the effluent solution showed that the neutral amino acids appeared in the same order as they occurred in the fractions passed into the column Fractions 105–117 (Fig 2b) contained serine and threonine only, and were bulked together This solution (1.21 l) was concentrated under reduced pressure and treated with a little charcoal The concentrated solution was then evaporated to dryness and yielded a mixture of serine and threonine as a colourless crystalline solid (3.93 g, yield on protein dry wt, 6.1%)

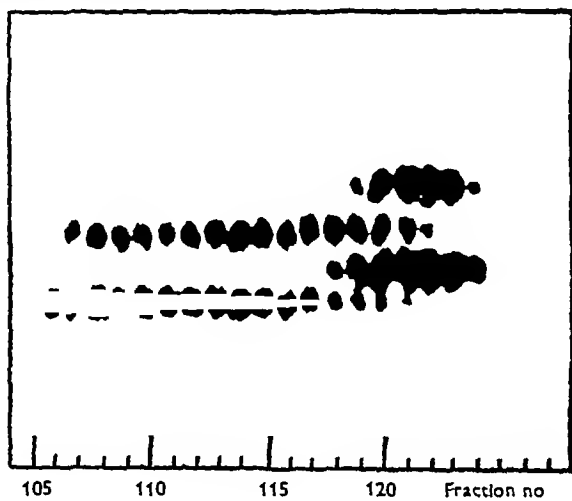


Fig 2a

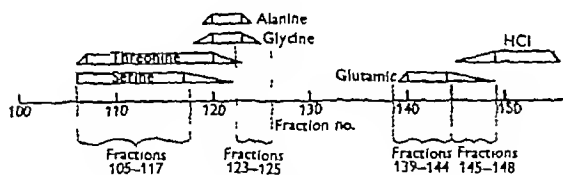


Fig 2b

Fig 2 Flowing chromatogram obtained by treating Band II, fractions 15–31 (Fig 1), on a column of De Acidite B Displacement developer 0.5N HCl (a) Paper chromatogram carried out on fractions from the flowing chromatogram (b) Key to amino acids shown in Fig 2a Glutamic acid was retained by the column

The glutamic acid contained in Band II was recovered from the column by displacement with 0.5N HCl following the procedure previously described (Part 2, p 518) The chromatogram illustrated in Fig 2b shows that part of the glutamic acid appeared in the free state (fractions 139–144) and part as the hydrochloride (fractions 145–148) The mixed solutions from these two fractions were concentrated separately, and, after treatment with a little charcoal, were evaporated to dryness and yielded glutamic acid (4.39 g) and glutamic acid hydrochloride (2.2 g) The combined yield (calculated as free glutamic acid) represented 9.5% of the protein (dry wt) The free glutamic acid had N, 9.6% (calc for $C_6H_9O_4N$, 9.5%)

Band III glycine and alanine Fractions 123–125 from the De Acidite column (Fig 2b) and fractions 32–36 from the Zeo Karb column (Fig 1b) were combined and yielded a cut containing glycine and alanine only This was concentrated to small bulk, evaporated to dryness *in vacuo*, and yielded the mixture of the two amino acids as a colourless crystalline solid (3.48 g, yield on protein dry wt, 5.4%)

Band IV valine and proline The valine proline band was narrow and consequently was too overlapped to allow its isolation from the flowing chromatogram obtained by a single passage through the column A cut was, therefore, taken which included the overlapping portions of Band III and Band V (fractions 37–46 inclusive, Fig 1b) The fractions were passed, in order, to a column of Zeo Karb 215, 36 cm in

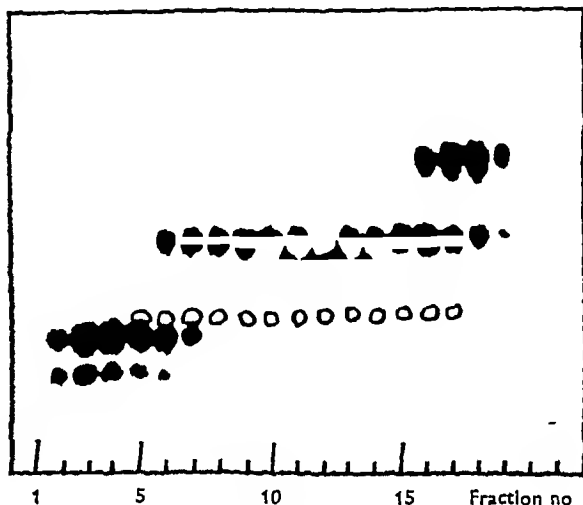


Fig 3a

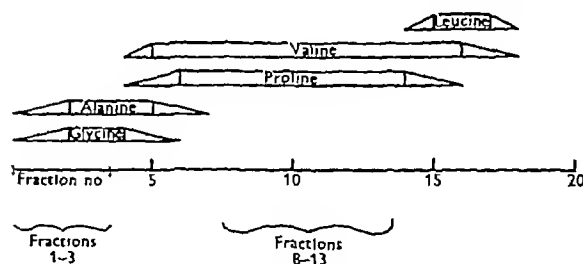


Fig 3b

Fig 3 Flowing chromatogram obtained by treating Band IV, fractions 37–46, (Fig 1), on a narrow column of Zeo Karb 215 Displacement developer 0.15N NH_3 (a) Paper chromatogram carried out on the fractions from the flowing chromatogram using butanol acetic acid water as the solvent (b) Key to amino acids shown in Fig 3a

height and 1.8 cm in diameter The column contained 40 g (dry wt) of the resin (60–80 mesh/in) and was prepared exactly as described for the longer column

The chromatogram obtained on displacement with 0.148N NH_3 is reproduced in Fig 3a In this chromatogram the valine proline band was much longer, and a centre portion was cut (fractions 8–13 inclusive, Fig 3b) which contained valine and proline only This, on evaporation, yielded the mixture as a colourless crystalline solid, 2.91 g (yield on protein dry wt, 4.5%) It will be observed (Fig 3b) that fractionation on the narrower column also resulted in the

separation of a further quantity of glycine and alanine. Fractions 1-3 contained glycine and alanine only, and yielded 0.71 g of the mixture as a crystalline solid which was suitable for addition to the material obtained from Band III (yield on protein dry wt, 1.1%).

Band V methionine, cystine and the isomeric leucines. Fractions 52-58 (inclusive) were mixed to give a solution containing the amino acids of Band V together with a trace of valine. The solution on evaporation to dryness yielded a crystalline product 5.2 g (yield on protein dry wt, 8.1%). The isolation of the leucines was of particular interest, and since the remaining components of the mixture, cystine and methionine, are both readily oxidized it was clear that a possible method of carrying out the isolation would lie in treating the mixture with a mild oxidizing agent in order to modify the properties of the two sulphur containing amino acids.

The crystalline mixture from Band V (5.0 g) was dissolved in water (1 l) and cooled by immersion in ice water. Bromine water was added until a permanent colour was obtained, and the slight excess of bromine was removed by passing a current of air through the solution under reduced pressure.

A paper chromatogram showed that the cystine had been fully oxidized to cysteic acid, while the methionine gave rise to a well characterized compound, presumably the sulfoxide, which had R_F values in various solvents that were identical with those given by the corresponding compound prepared from an authentic sample of methionine. Cysteic acid was then removed from the solution by passing it through a column containing De Acidite B (cf. Consden, Gordon & Martin, 1948; Partridge & Westall, 1949). The

of a dilute solution of ammonia. A chromatographic separation of this kind is particularly suited to serve as the basis of a systematic procedure for the isolation of amino acids on a preparative scale since, by one passage through the column, the complicated mixture of amino acids may be broken down into six or seven simple fractions, each in a form suitable for further fractionation.

The means to be adopted for the isolation of pure amino acids from the simplified mixtures resulting from first passage through the column must depend to a great extent upon the personal choice of the investigator since the aim is usually to prepare only a few of the amino acids occurring in the protein, and it is often possible to obtain a good yield of one component by sacrificing another. With this in mind, it appears probable that the classical methods of separation—fractional crystallization either of the free amino acids or of their derivatives—would offer the most direct approach. However, where the isolation of all or most of the amino acids is equally desired, as may often be the case in experiments with labelled amino-acids, it is clear that chromatographic methods for completing the separation would be particularly desirable.

It has already been pointed out that, in the initial passage through the cation exchange column, the bases and amino acids separate into bands comprising components of similar affinity for the ion exchanger. This 'affinity' is a complicated function in the case of the amino acids since they are ampholytes. All that should be necessary to secure the further separation of two components comprising a band, is to refractionate the band under new experimental conditions such as to modify differentially either the basic or the acidic ionization of the components.

Some of the possible variations in experimental conditions that may be applied to induce further separations are as follows: (1) The use of a water soluble organic solvent such as ethanol or acetone in order to modify the ionization of the carboxylic acid groups in the amino acids. (2) The utilization of variations in temperature or concentration. (3) The choice of a solvent mixture having a solvent water partition in favour of the amino-acids it is desired to separate.

To these methods two further may be added: (4) The choice of a second ion exchanger with different properties from that effecting the first separation (e.g. the use of an anion-exchange column to follow a separation carried out on a cation exchange column). (5) The properties of one or more of the components of the mixture may be modified by chemical means.

The effect of an organic solvent in differentially modifying the ionization of a pair of amino acids (separation of type 1) may be illustrated by an

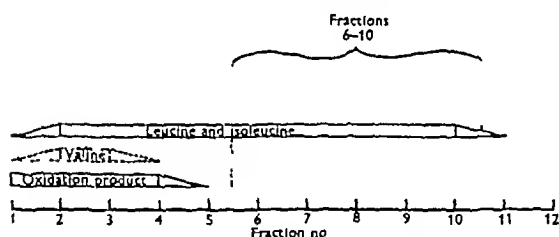


Fig. 4. Flowing chromatogram given by fractions 52-58 (Fig. 1) after mild oxidation with bromine water and removal of cysteic acid. Displacement from a column of Zeo Karb 215 with 0.154N-NH₃.

remaining solution was then further fractionated on a column containing Zeo Karb 215 (dry wt, 40 g, height 35 cm, diameter 1.6 cm) by displacement with 0.154N NH₃. The chromatogram obtained is shown in Fig. 4. Fractions 6-10 inclusive (Fig. 4) contained the isomeric leucines in a pure condition and on evaporation to dryness yielded colourless crystals 2.28 g (Found N, 10.5, calc for C₆H₁₃O₂N, 10.7, yield on protein dry wt, 3.6%).

DISCUSSION

The greater part of the data presented in this paper has been derived from the analysis of the flowing chromatogram obtained when the mixture of amino-acids resulting from the hydrolysis of a protein is displaced from a column of Zeo-Karb 215 by means

example, viz the isolation of glutamic acid from a mixture containing serine and threonine by use of 50 % aqueous acetone as the solvent phase in a displacement chromatogram. In one experiment the primary separation of a protein hydrolysate gave a mixed band comprising glutamic acid, serine and threonine. This band was cut so as to include these three amino acids together with a little aspartic acid. The mixed solution was then displaced from a second column of Zeo Karb 215 by means of a dilute solution of ammonia (0.05N) in 50 % aqueous acetone. The resulting chromatogram is illustrated in Fig 5 and shows a satisfactory separation of

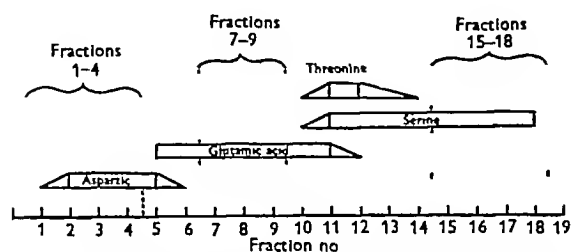


Fig 5 Flowing chromatogram given by the amino acids of Band II on displacement from a column of Zeo Karb 215 by means of 0.05N NH_3 in 50 % aqueous acetone

glutamic acid from the other components of the mixture, in addition there was a partial separation of serine from threonine. This latter effect was probably due to the intervention of solvent-water partition (separation of type 3). Since the solvent uptake of Zeo-Karb 215 varied considerably with the acetone content of the solution, the resin was first treated alternately with 2N- NH_3 and 2N-HCl in 50 % aqueous acetone before packing the column, and the acetone content of the solvent phase was thereafter maintained at the 1:1 ratio.

Separations of types 4 and 5 have already been reported in the text, but the possibilities of the various methods listed above have not been fully explored, and no doubt further work will bring to light other ways of taking advantage of variations in environment to secure the separation of an individual ion type from a solution containing related electrolytes.

SUMMARY

1 A systematic procedure is described for the isolation of amino acids from protein hydrolysates on a preparative scale.

2 Since tyrosine and phenylalanine interfere with chromatographic separation on ion-exchange resins, these were first removed, together with soluble humin, by adsorption on charcoal. Tyrosine and phenylalanine were recovered by eluting the charcoal with aqueous phenol-acetic acid.

3 In the example given the charcoal-treated hydrolysis product of commercial egg albumin was fractionated on a column of Zeo-Karb 215 by displacement with ammonia solution.

4 The flowing chromatogram so obtained was analyzed by filter-paper chromatography. The results showed that the mixture had been resolved into seven bands: I, aspartic acid; II, glutamic acid, serine and threonine; III, glycine and alanine; IV, valine and proline; V, leucine, isoleucine, methionine and cystine; VI, histidine and an unidentified amino-acid; VII, lysine.

5 The amino acids or amino acid mixtures contained in each band were recovered as salt-free crystalline solids by evaporating appropriate fractions from the effluent solution. The crystalline products obtained represented a yield of 45.6 % calculated on the weight of dry protein.

6 The isolation of individual amino acids by further fractionation of the simplified mixtures contained in the bands is discussed. As examples of the procedures suggested, three such separations are described: (a) glutamic acid was isolated from Band II by adsorption on De-Acidite B; (b) a mixture of the isomeric leucines was isolated from Band V by selective oxidation with bromine water, followed by further fractionation on ion exchange columns; (c) the separation of glutamic acid from Band II was carried out by displacing the mixture from a column of Zeo-Karb 215 by use of a dilute solution of ammonia in 50 % aqueous acetone.

The experimental work recorded in this paper was carried out by Mr R. G. Westall and Mr R. C. Brimley. The partition chromatograms were performed by Mr D. F. Elsdon and the photography by Mr D. P. Gatherum. The work forms part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Distribution of Urethane in Animal Tissues, as Determined by a Microdiffusion Method, and the Effect of Urethane Treatment on Enzymes

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The narcotic action of urethane has been known for a long time (Schmiedeberg, 1885), and the leucopenic action was described by Hawkins & Murphy (1925) almost 20 years before its first clinical use in treatment of leukaemia (Paterson, ApThomas, Haddow & Watkinson, 1946). Urethane has a relatively slow toxic action on cell nuclei causing pycnosis. It is a radiomimetic poison like trypanflavine and the nitrogen mustards (Dustin, 1947), and causes chromosome breaks (Boyland & Koller, 1949) when given in larger doses. This radiomimetic action may be associated with the induction by urethane of lung tumours (Nettleship & Henshaw, 1943).

The nature of the biochemical lesions underlying the narcotic and radiomimetic effects is still unknown, although Fisher & Henry (1944) suggest that the respiration associated with activity rather than that concerned with the basal metabolism is primarily inhibited. Meyerhof & Wilson (1948) have shown that phenylurethane inhibits hexokinase of mammalian cells.

An improved method of estimating urethane in tissues is described in this paper, and this has been used to study the distribution and excretion of urethane in animals. Urethane is hydrolyzed by caustic alkalis to yield ethanol and this reaction is the basis of the method of estimation described by Archer, Chapman, Rhoden & Warren (1948), and the present method.

EXPERIMENTAL

Preliminary trials showed that urethane was hydrolyzed at room temperature and at 37° by KOH, and the rate of this reaction in Conway units with different concentrations of alkali was followed by the method described by Winnick (1942) for estimation of the ethanol formed.

The course of the reaction, shown in Table 1, is such that the reaction is only complete in 16 hr if the final concentration of KOH is 2N. If higher concentrations of alkali are used with whole blood, precipitation occurs and the results are irregular. Urethane is not decomposed by K_2CO_3 under these conditions and for this reason K_2CO_3 was added to the blank unit in carrying out determinations.

Method of estimation. A standard volume of 1 ml 0.04N $K_2Cr_2O_7$ in 10N H_2SO_4 is placed in the central compartment of a Conway unit. The sample, which should be less than 0.7 mg urethane, contained in 1 ml fluid, and 1 ml 4N-KOH, are placed in the outer ring of the unit. A blank is set up with a 1 ml sample of urethane and 1 ml 2N K_2CO_3 . The alkalis used must not contain volatile reducing substances and are kept in glass stoppered bottles. The units are sealed and incubated at 37° for 16 hr or more. At the end of the incubation KI is added to the acid $K_2Cr_2O_7$, and the liberated I_2 titrated with 0.02N $Na_2S_2O_3$. The difference in titre between the two units is proportional to the amount of urethane present, 1 ml 0.02N- $Na_2S_2O_3$ is equivalent to 0.445 mg urethane.

Table 1. *Hydrolysis of urethane at 37°*

(Yield of ethanol as % of theory at different times)

Alkali used	KOH								K_2CO_3	
	Final conc. in outer ring								M	
Urethane present (mg)	5N	2N	N	0.5N	5N	2N	N	0.5N	0.25	0.5
	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.5
Time (hr)	1	40	—	—	—	—	—	—	—	—
	2	70	19	13	8	8	8	0	0	0
	4	101	83	58	67	56	12	23	4	0
	8	101	85	76	74	67	12	33	0	0
	16	—	100	98	84	92	80	75	0	0
	32	—	98	95	98	100	92	85	0	0

Alternatively, the titration can be carried out with a Conway burette using 0.1N $Na_2S_2O_3$. With this modification 1 ml of 0.025N $K_2Cr_2O_7$ in 10N H_2SO_4 is placed in the central compartment and the urethane sample (e.g. 0.1 ml blood) is incubated with 2 ml of M K_2CO_3 as the blank, and a further 0.1 ml blood treated with 2 ml 2N-KOH in the outer ring of the second unit. Thus by using a sensitive microburette the estimation can be carried out on 0.2 ml blood. The amount of urethane and volatile reducing matter in the sample must not be more than the equivalent of 0.5 mg urethane.

Duplicate estimations agreed within 5% and the method could be applied directly to blood, urine or tissue extracts deproteinized with tungstic acid or trichloroacetic acid. The distillation method could not be used with urine and some extracts on account of the frothing which occurred. Determinations on normal human blood and on blood of normal rats and rabbits gave no indication of the presence of any substance reacting as urethane in this reaction.

RESULTS

Excretion of urethane Groups each of six rats kept in metabolism cages were given intraperitoneal injections of 500 mg urethane/kg body weight, as a 5 % (w/v) solution. The urine was collected and the urethane content determined. No urethane was found in the urine of control animals, which were injected with 10 ml water/kg body weight. Results of a typical experiment are shown in Table 2. The injected animals excreted more urine than the controls so that the drug had a diuretic action, but only about 4% of the injected urethane was excreted in urine.

Table 2 *Excretion of urethane by rats*

(Six rats injected with 500 mg urethane/kg body wt in 5% (w/v) solution. Six control rats injected with 10 ml water/kg body wt.)

Time after injection (hr)	Vol of urine collected		Total urethane excreted (mg/kg body wt)	Urethane excreted (percentage of dose)
	Controls (ml.)	Treated (ml.)		
1	Nil	2.5	1.0	0.3
2	0.5	8.0	6.9	1.4
3	0.5	1.8	8.1	1.6
6	2.0	3.0	10.1	2.0
24	4.0	25.0	20.4	4.1
Total urine	7.0	40.3	—	—

Distribution of urethane in tissues Rats were injected subcutaneously with 1 g urethane/kg body weight, and killed at different times after injection. A specimen of blood was taken and organs were removed and extracted with 2 vol of 10 % trichloroacetic acid. Urethane was estimated in the blood and in the organ extracts.

Deproteinized extracts of rat liver, but not of other tissues, prepared with trichloroacetic acid, metaphosphoric acid or tungstic acid, produced volatile reducing substances on standing. The nature of this material was not determined. Determinations carried out on fresh liver extracts appeared to be quite satisfactory.

The results shown in Table 3 indicate that in normal rats the urethane of a dose of 1 g/kg disappears from the body within 24 hr. As very little urethane is excreted in urine and none could be detected in faeces most of the drug must be metabolized. The metabolism is very much slower in tumour-bearing rats, in which some urethane remains even after 48 hr.

Observation of the behaviour of rats indicated that the threshold concentration of urethane for narcosis was between 60 and 80 mg/100 ml blood. Rats with blood concentrations of 60 mg/100 ml or below were not anaesthetized, while those with concentrations of 80 mg/100 ml or over were anaesthetized. The same dose of urethane produced much

more prolonged narcosis in tumour-bearing than in normal rats. A dose of 1 g/kg body weight induced anaesthesia for 8–12 hr in normal rats, but for 24 hr in tumour-bearing rats.

Table 3 *Urethane content of rat tissues after injection of 1 g/kg body weight*

Time after injection (hr)	A Normal rats			
	Urethane present (mg/100 g)			
	Blood	Brain	Liver	Spleen
1	140	138	—	—
2	100	85	100	110
5	90	—	80	90
18	57	75	—	40
24	0	0	2	2
40	0	0	0	0
80	0	0	0	0

Time after injection (hr)	B Rats with Walker carcinoma	
	Urethane present (mg/100 ml)	
	Blood	Tumour
1.5	100	100
2	75	90
5	93	80
18	110	65
20	120	110
24	77	55
40	32	15
49	10	25
55	7	0

The injected urethane was rapidly distributed in the organs of rats or rabbits with no indication of localization of the drug in any one organ or tissue.

Urethane in blood of patients The method described was used for estimation of urethane in blood from patients under treatment with urethane. The blood was collected in tubes containing fluoride. The highest concentration found in human blood was 59 mg/100 ml in a leukaemia patient receiving 7 g urethane daily. This patient was sleepy, but not anaesthetized, which suggests that the concentration of urethane inducing narcosis is higher in man than in rats. Another leukaemia patient who was given 6 g urethane daily had blood levels of 23, 19 and 40 mg/100 ml on samples taken at weekly intervals. Patients dosed with 3 g urethane daily had blood concentrations of 10, 12, 12, 14, 16, 18 and 19 mg/100 ml.

Blood cholinesterase in patients treated with urethane As many potent inhibitors of cholinesterase are urethane derivatives, the continuous treatment with urethane might cause reduction in blood cholinesterase. The esterase activity of the plasma with acetylcholine, benzoylcholine and acetyl-β-methylcholine as measured by liberation of carbon dioxide from sodium bicarbonate in the Warburg apparatus (following the methods of Hawkins & Gunter, 1946) are shown in Table 4. The

Table 4 *Esterase activity of human blood constituents under urethane treatment*

(Subjects received 3 g/day by mouth)

 CO_2 ($\mu\text{l/hr}$) evolved by 1.0 ml plasma or 1.0 ml washed whole erythrocytes in presence of 0.025 M NaHCO_3 at 37°

	Substrate	Plasma			Erythrocytes	
		0.06 M-Acetylcholine bromide	0.06 M-Benzoylcholine chloride	0.03 M-Acetyl β -methylcholine chloride	0.06 M-Acetylcholine bromide	0.03 M-Acetyl β -methylcholine chloride
Mr E						
	1 day before treatment	4750	1765	312	1170	1295
	2 days after beginning of treatment	4550	2100	240	810	1074
	5 " " " "	4685	1020	585	1780	1270
	11 " " " "	4230	—	280	800	1015
	13 " " " "	4010	—	300	1000	1067
	20 " " " "	3350	1280	140	1400	1100
Mrs A						
	4 days before treatment	1805	600	135	2700	1960
	2 hr " " "	1840	480	175	1180	—
	7 days after beginning of treatment	1030	572	100	1370	1430
	9 " " " "	1371	554	114	—	1600

results indicate that urethane treatment causes a decrease in the ability of the plasma to hydrolyze acetylcholine. In one case (Mr E) this seems to be due to a fall in pseudocholinesterase as indicated by the decreased hydrolysis of benzoylcholine. In the second series (Mrs A) the plasma showed a fall in true cholinesterase, as the samples taken during treatment hydrolyzed acetyl- β -methylcholine more slowly.

Effect of urethane on tissue metabolism. Urethane given to tumour bearing rats or mice in doses sufficient to inhibit mitosis had no inhibitory effect on respiration or glycolysis of tissue slices as measured in the Warburg apparatus (Table 5). Treatment with urethane caused a slight increase in respiration of tumour tissue. The doses of urethane given would be

sufficient to induce cancer of the lung, and did inhibit mitosis of the jejunal mucosa and Walker rat carcinoma as shown by examination of histological preparations of tissue from animals used for the manometric measurements. The glycolysis and respiration of mouse lung and jejunum and of Walker carcinoma tissue from rats were not affected by 0.011 M-urethane added *in vitro*. This concentration is equivalent to that which produces cytological effects in these tissues *in vivo*.

DISCUSSION

The results show that urethane disappears from the body of normal rats for the most part without being excreted. It is presumably metabolized. Experiments carried out with urethane containing ^{14}C in the carbamate radical by Skipper, Bryan, White & Hutchison (1948) have shown that very little of the carbamate is excreted in urine, and that about 90% of the radioactivity passes into the respiratory gases in 24 hr.

The work with radioactive urethane also supports the findings in the present paper, that there is no accumulation of urethane in any particular organ and that the breakdown is slower in tumour-bearing animals than in normal animals (Skipper, personal communication). The reduced metabolism of urethane in tumour-bearing rats is not due to retention in the tumour, but is probably due to deficiency of enzymes concerned in the breakdown of the urethane molecule. This would be comparable with the reduced content of other enzymes such as catalase (Greenstein, 1943; Weil Malherbe & Schade, 1948), D-amino acid oxidase (Westphal, 1943), or arginase (Greenstein, Jenrette, Mider & White, 1941) in the livers of animals with tumours.

Table 5 *Effect of urethane on tissue metabolism in vitro*

(Measurements during 1 hr made in the appropriate Krebs Ringer solution with Warburg manometers at 38° . Q_{O_2} in O_2 and $\text{Q}_{\text{CO}_2}^{\text{N}}$ values in $\mu\text{l/mg}$ dry wt/hr.)

Tissue	Tissue from normal animals without addition		Tissue from animals treated with urethane		Tissue from normal animals with 0.011 M urethane	
	$\text{Q}_{\text{CO}_2}^{\text{N}}$	Q_{O_2}	$\text{Q}_{\text{CO}_2}^{\text{N}}$	Q_{O_2}	$\text{Q}_{\text{CO}_2}^{\text{N}}$	Q_{O_2}
Mouse lung	4.2	10.2	3.9*	7.5*	4.2	11.0
Mouse jejunum (whole wall)	12.6	19.2	16.7*	—	11.2	18.3
Rat Walker carcinoma	29.0	9.2	28.9†	9.9†	28.7	9.9
Rat-jejunal mucosa	5.35	—	4.95†	—	—	—
Rat-kidney medulla	25.4	—	25.9†	—	—	—
Rat-brain homogenate	7.0	—	6.1†	—	—	—

* Mice fed on diet containing 2% urethane for 3 days before measurements.

† Rats given doses of 1 g urethane/kg body wt, on 3 successive days before measurements.

The absence of inhibitory effect of urethane (given in doses which reduce mitosis) on tissue metabolism indicates that urethane possibly inhibits some specific cell process or prevents the utilization of the energy of glycolysis or respiration from synthetic processes. Studies on the effect of methyl-di-(2-chloroethyl)-amine (HN2) on the metabolism of the Walker carcinoma showed a decrease in glycolysis some days after treatment (Boyland, Clegg, Koller, Rhoden & Warwick, 1948). Urethane resembles nitrogen mustard in its cytological effects, and a similar effect on metabolism had been expected. The inhibition of glycolysis observed with nitrogen mustard is probably due to effects on hexokinase, and this may not be the fundamental cause of the nuclear damage, but rather an indication that phosphokinases are affected. Urethane may inhibit such phosphokinases (as yet unidentified) which are concerned with nucleoprotein metabolism, but the biochemical mechanism of its action is as yet unknown.

SUMMARY

1 As urethane is hydrolyzed by cold caustic alkali to give quantitative yields of ethanol, it can be

estimated by incubation with potassium hydroxide in Conway units and determination of the ethanol absorbed in acid potassium dichromate. This method can be applied directly to blood (using 0.2 ml), urine or tissue extracts.

2 Following injection, urethane becomes evenly distributed in all the tissues of the body. Only a small amount is excreted in urine but the greater part is metabolized. The metabolism is slower in tumour-bearing rats than in normal rats.

3 Of two patients treated with urethane the blood of one showed a decrease in true cholinesterase while the blood pseudocholinesterase of the other patient decreased.

4 Treatment of rats or mice with urethane in doses sufficient to cause inhibition of mitosis or induce cancer of the lung did not reduce the respiration or glycolysis of lung, jejunum or Walker carcinoma tissue.

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An Immune Globulin Fraction from Bovine Precolostrum

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This paper describes the preparation and partial characterization of an immune globulin fraction from bovine precolostrum. This material was studied as part of a joint programme on diseases of young calves because of its possible relation to colostrum, and the role of the latter in the transference of passive immunity to the suckling animal.

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Bovine precolostrum is a viscous honey-like substance obtained from the udders of pregnant heifers at about half term. It was first described by Woodman & Hammond (1923) and Asdell (1925), who showed that it was deficient in the normal constituents of milk, but very rich in globulin. They suggested that it might prove rich in antibodies, a point recently demonstrated by Blakemore (1947), and that its accumulation in the udder well before

calving and its subsequent dilution by a true lacteal secretion would account for the composition and properties of colostrum and the rapid change from colostrum to milk after calving

Colostrum itself was shown by Orcutt & Howe (1922) to be rich in easily salted-out globulins and antibodies, both of which were absent from the serum of the newborn calf, but appeared therein after the ingestion of colostrum. This work explained the experiments of Smith & Little (1922), which showed that feeding colostrum prevented losses of calves from infections in the first week of life, and suggested

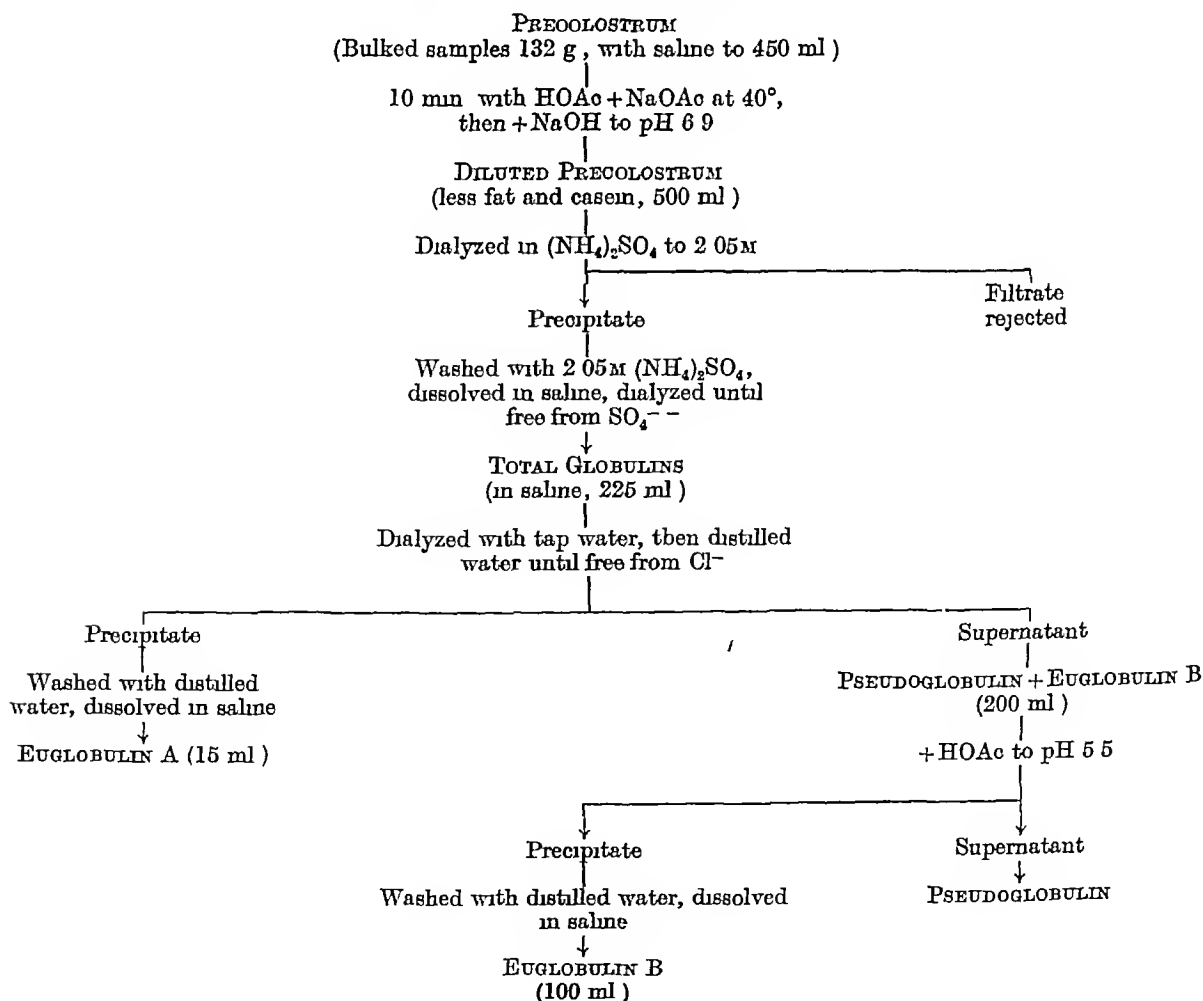
that one of the most important constituents of colostrum was an immune globulin. Such a protein has recently been prepared from this source and investigated by Smith (1946*a, b*)

An immune globulin from bovine precolostrum has been investigated in the present work. This secretion has been examined for Howe's (1922) nitrogen fractions, bulked samples fractionated, the fractions examined for the distribution of antibody and the properties of an immune globulin fraction investigated, mainly by physico chemical methods

Table 1 *Nitrogen fractions in bovine precolostrum*
(Fractionation by a modification of Howe's (1922) method)

Sample	Nitrogen (g /100 ml)						
	Total	Globulin	Casein	Albumin + non protein	Euglobulin	Pseudo globulin I	Pseudo globulin II
Heifer 7	4.72	3.61	0.62	0.49	1.10	2.12	0.39
Heifer 16	3.62	2.75	0.50	0.37	1.85	0.89	0.01
Bulk I	5.04	4.60	0.20	0.23	3.17	1.23	0.20
Bulk II (diluted)	2.23	2.05	0	0.18	1.31	0.70	0.04

Table 2 *Fractionation, method I*



EXPERIMENTAL AND RESULTS

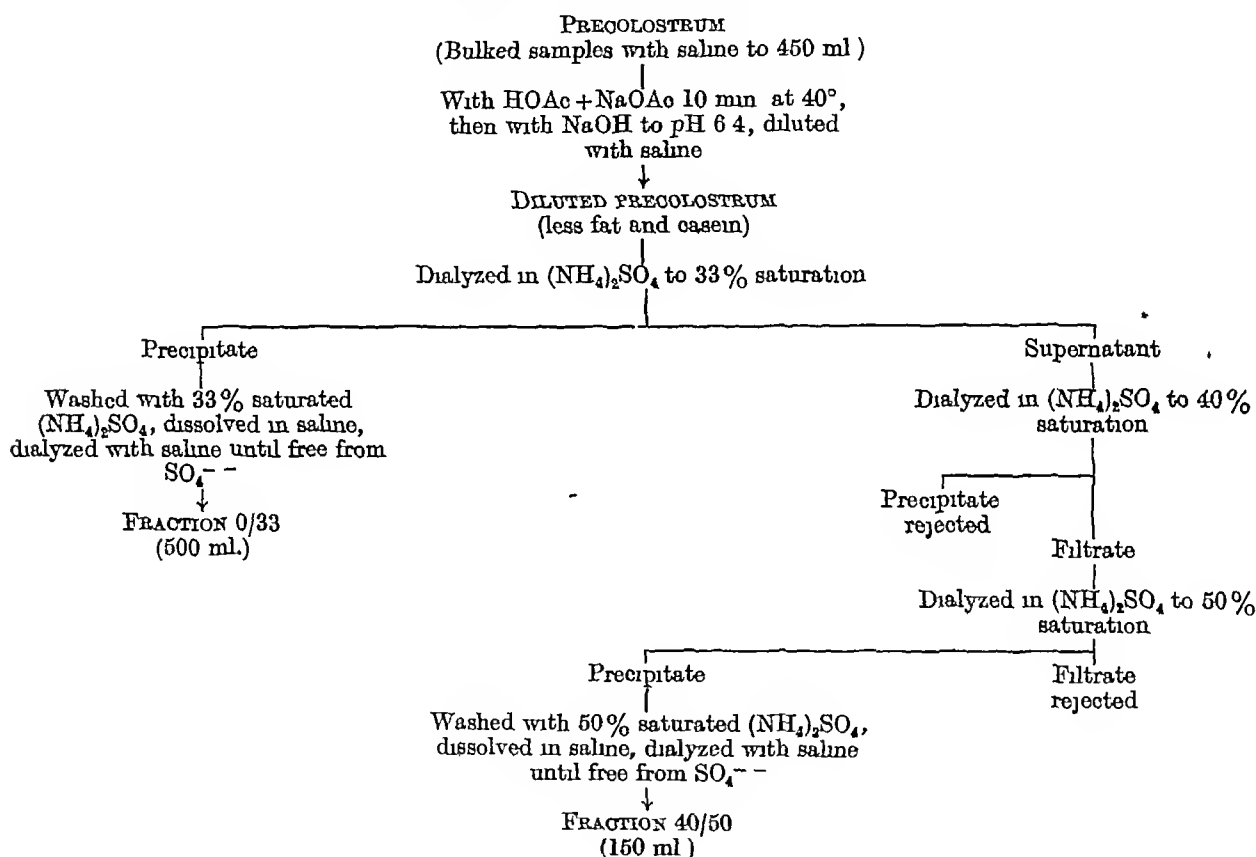
Nitrogen fractions

It has been noted that bovine colostrum is rich in easily salted-out globulins, viz the euglobulin and pseudoglobulin I fractions of Howe. Accordingly, to see how the proteins of precolostrum compared with those of colostrum in this respect, the distribution of nitrogen in different fractions was investigated in four samples of precolostrum using a modification of Howe's (1922) method

Table I shows that of the globulin N, which forms the greater part of the total N, a high proportion belongs to the more easily salted out euglobulin and pseudoglobulin I fractions. In this respect it resembles the globulin N of bovine colostrum. If the antibodies in the two secretions have similar salting out limits, then precolostrum should be a good source of an easily salted out immune globulin fraction.

Bulk fractionation

Two lots of bulked samples of precolostrum, bulk I and bulk II (diluted) of Table I, were accumulated for fractionation. The methods of fractionation employed depended on differences of solubility in

Table 3 *Fractionation, method II*

The globulin was divided into euglobulin, pseudoglobulin I and pseudoglobulin II fractions by precipitation with 14, 18 and 22% (w/v) Na_2SO_4 at 34° . Casein, however, was estimated by acidification of the dilution in saline used for the total N estimation with acetic acid, and not by acidification of the filtrate from 14% (w/v) Na_2SO_4 , as in the original procedure, allowance was made for its precipitation by 18% (w/v) Na_2SO_4 in calculating pseudoglobulin I. This modification seemed justified as, on comparison of the two procedures in the analysis of precolostrum, the former gave lower values than the latter, and this suggested that proteins additional to casein were precipitated when the original method was used. Consequently, the present values for casein are slightly lower and for pseudoglobulin I slightly higher than would otherwise have been the case.

water and concentrated ammonium sulphate solutions respectively and are given schematically in Tables 2 and 3.

In both methods the bulked samples were first diluted in about 450 ml saline, any fat and casein were removed by acidification, and the pH readjusted to approximately neutral. $(\text{NH}_4)_2\text{SO}_4$ was introduced by dialyzing in the calculated amount of salt from a rotating cellophane sac, and precipitates were removed by centrifugation.

In the first method the total globulins were separated at 2.05M $(\text{NH}_4)_2\text{SO}_4$, and dialyzed against water to give fraction euglobulin A, the supernatant was adjusted to pH 5.5 and separated into euglobulin B, and pseudoglobulin fractions. The euglobulin fractions were finally dissolved in saline.

In the second method the solution of precolostrum was brought to 33% saturation with $(\text{NH}_4)_2\text{SO}_4$ to give a precipitate of fraction 0/33, the supernatant was brought to 40% saturation and the precipitate rejected, the filtrate from this was then brought to 50% saturation to give a precipitate of fraction 40/50 the filtrate from this was rejected. The two fractions were dissolved in saline to give a rather turbid white solution (0/33) and a clear pink brown solution (40/50).

An approximate N balance sheet of both fractionation procedures was obtained by measuring the volume and N content of solutions at different stages of the procedure. The results are given in Table 4. In method I, the water soluble pseudoglobulin fraction accounted for 72% of the globulin initially separated and in method II, the readily salted out fraction 0/33 contained 74% of the total N in the starting material.

colostrum has been reported in two papers. Orcutt & Howe (1922) found an upper limit of 16.5 g/100 ml for the precipitation of antibodies to *Escherichia coli* and *Brucella abortus* by sodium sulphate, and Smith (1946*a, b*) prepared an immune globulin fraction by precipitation at 0.3 saturation with ammonium sulphate. The present antibody globulin of precolostrum therefore resembles that of colostrum in its salting out behaviour.

The 0/33 $(\text{NH}_4)_2\text{SO}_4$ fraction would be described as a euglobulin in Sørensen's terminology. However, it has been seen that the antibody was also found in the pseudoglobulin fraction obtained by dialysis. The terms euglobulin and pseudoglobulin therefore need some discussion. They have

Table 4. *Distribution of nitrogen and antibody in fractionation experiments*

Fraction	Approx vol (ml)	N content of fraction				Titre to <i>Brucella</i> <i>abortus</i>
		(g /100 ml)	Total in fraction (g)	As % of globulin N	As % of total N	
Fractionation method I						
Dilute precolostrum	450	1.48	6.65	—	—	320
Dilute precolostrum less fat and casein	500	1.14	6.03	—	—	320
Total globulins	225	1.35	3.36	—	50.5	320
Euglobulin <i>A</i>	15	0.06	0.01	0.3	0.15	Absent
Pseudoglobulin + euglobulin <i>B</i>	200	1.20	3.00	—	—	320
Euglobulin <i>B</i>	100	0.40	0.57	17.0	8.6	Absent
Pseudoglobulin	175	0.97	2.42	72.0	36.9	320
Albumin, casein and losses	—	—	3.65	—	—	—
Fractionation method II						
Dilute precolostrum	450	2.23	10.0	—	—	—
Fraction 0/33	500	1.40	7.4	—	74	320
Fraction 40/50	150	0.16	0.25	—	2.5	Absent
Albumin, casein, fraction 33/40 and losses	—	—	2.35	—	23.5	—

Distribution of antibody

The agglutinin to *Brucella abortus* is one of the antibodies that have been studied in bovine colostrum. This is because it is a convenient antibody for many purposes since it occurs frequently in this species and can be easily titrated, and not because it is of any importance to the newborn calf. This antibody was known to have been present in the bulk samples of precolostrum used in the present work. Its distribution in the various fractions obtained above was therefore determined as a guide to the nature of the globulin of this and possibly of other antibodies in the secretion. As shown in Table 4, it was present in the pseudoglobulin fraction obtained by dialysis in method I and in fraction 0/33 obtained by salting out in method II and was absent from the remaining fractions.

The distribution of antibody in the ammonium sulphate fractions shows that the antibody globulin was a readily salted-out globulin. This property provides a broad basis for comparison with other proteins that are possibly related. The salting-out behaviour of the immune globulins of bovine

been applied to the water insoluble and water soluble fractions obtained by dialysis, to proteins precipitating in the ranges 0–33 and 33–50% saturation with $(\text{NH}_4)_2\text{SO}_4$, to proteins precipitating in the ranges 0–14 and 14–22% Na_2SO_4 respectively and to fractions obtained by precipitation at different concentrations of ethanol. It is possible to adjust the conditions of these precipitations to yield quantitatively similar results, but this in itself is insufficient proof that the fractions obtained by the different methods are qualitatively the same or are definite chemical entities. In fact, both fractions obtained by dialysis of serum proteins have been shown to be electrophoretically pure, disperse, and salting out methods have yielded fractions containing a single electrophoretic component which can be further separated into water soluble and water insoluble fractions by dialysis. It is evident from the present results that antibody globulin cannot be described as an euglobulin or a pseudoglobulin without qualifying the term by the conditions of separation.

Electrophoretic examination of fractions 0/33 and 40/50 in solution

As the relation between protein fractions obtained by dialysis and the original protein is uncertain, and as salting out methods have been used successfully

in separating serum globulins, further examination of the fractions described in the preceding section was limited to those obtained by salting out with ammonium sulphate. These fractions were examined by electrophoresis in the Tiselius apparatus to obtain information on their purity and to enable some comparison to be made with other proteins.

The two fractions, were examined at 4° using a simple schlieren optical system, 1 month after preparation, and at

20° using the Philpot diagonal schlieren optical system, 7 months after storage at 4°. The velocity of each component was obtained either graphically by following its migration with a ground glass scale or a cathetometer, or by measuring photographs taken near the start and finish of the electrophoresis, or by both methods.

The number and proportion of the chief components were similar at the two temperatures, but the mobilities were appreciably higher at 20° than at 4° (see Table 5 and Fig. 1). At 4°, fraction 0/33 showed a single strong band with a

Table 5 Electrophoretic examination of fractions 0/33 and 40/50 in the Tiselius apparatus

Temperature	4°				20°			
Fraction	0/33		40/50		0/33	40/50		
Buffer	Phosphate		Borate		Phosphate	Phosphate		
pH	7.0		8.5		7.0	7.5		
Ionic strength	0.1		0.06		0.1	0.1		
Protein concentration (g/100 ml)	1.0		1.0		1.0	1.5		

Component	Mobility (μ/sec/V/cm)									
	Asc	Desc	Asc	Desc	Asc	Desc	Asc	Desc	Asc	Desc
1	—	—	—	—	—	—	—	—	1.61	1.37
2	—	—	—	—	1.66	1.37	—	—	1.27	1.03
3	—	—	—	—	1.02	0.93	—	—	1.20	—
4	—	—	—	—	0.51	0.50	—	—	1.02	0.84
5	0.38	0.40	0.45	0.37	0.40	0.34	0.74	0.54	0.88	—
6	0.20	0.16	0.23	0.24	0.26	0.23	0.41	0.36	0.65	0.50
7	—	—	—	—	0.14	—	—	—	—	—
Chief component	6		4		4		6		4	
Coloured component	5		4		4		5		—	

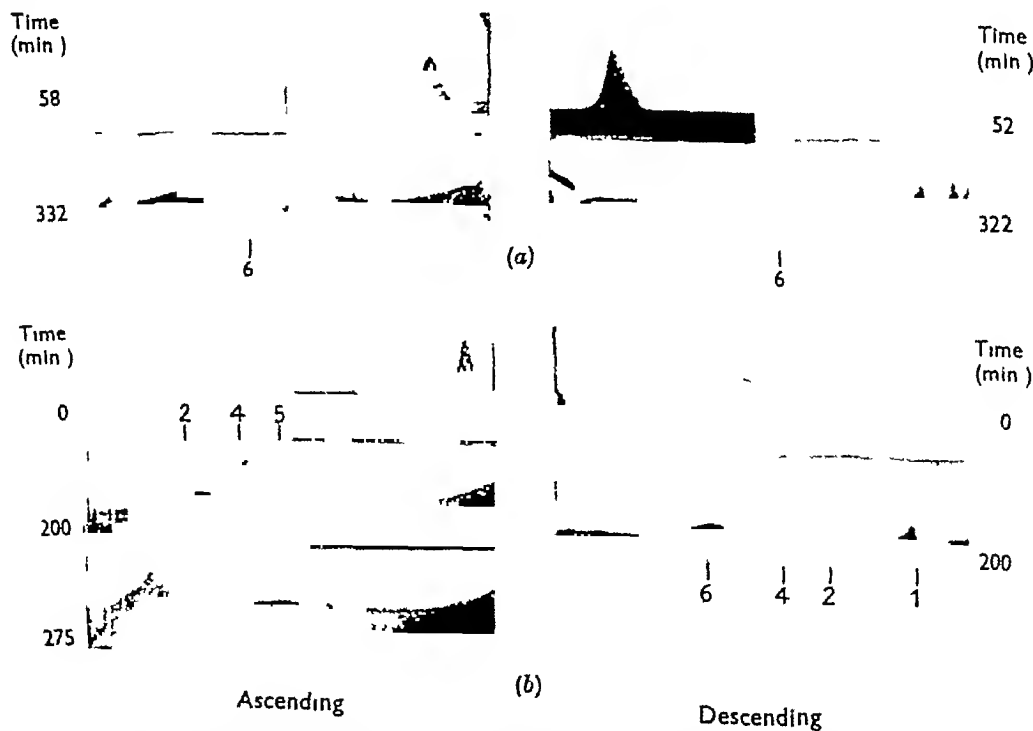


Fig. 1 Electrophoresis of protein fractions in phosphate buffer at pH 7.5 and 20°. (a) fraction 0/33, 1.5 g/100 ml, $E=4.3$ V/cm, (b) fraction 40/50, 1 g/100 ml, $E=3.0$ V/cm. Nos. 1, 2, 4, 5, 6 in the diagram indicate components.

mobility of approximately $0.2 \mu/\text{sec}/V/\text{cm}$ at pH 7.0 in phosphate buffer and at pH 8.5 in borate buffer, this band became rather diffuse at the end of a day's run a second component with a mobility of $0.4 \mu/\text{sec}/V/\text{cm}$ was detected by its brownish pink colour, but was not present in sufficient concentration to give a band. Fraction 40/50 showed six bands, some of them only just discernible the chief component, no. 4, had a mobility of approximately $0.5 \mu/\text{sec}/V/\text{cm}$ at pH 7.0 in phosphate buffer the pink colour appeared to be present and to move at the same rate.

At 20° , fraction 0/33 showed one main peak with a mobility of approximately $0.4 \mu/\text{sec}/V/\text{cm}$ at pH 7.5 in phosphate buffer, which became squat towards the end of the day's run. The coloured substance could be seen but gave no peak, it had an average mobility of $0.6 \mu/\text{sec}/V/\text{cm}$. A number of small peaks were visible which were probably due to convectional disturbances. Fraction 40/50 again showed six components, the chief component, no. 4, had a mobility of approximately $0.9 \mu/\text{sec}/V/\text{cm}$ at pH 7.5 in phosphate buffer.

The differences in mobilities in the two sets of experiments are greater than would be expected to arise from the small differences in pH of the measurements, as the latter were made on the alkaline side of the isoelectric point where the slope of the pH mobility curve of proteins is least. They are probably due to the effect of temperature on viscosity, since an approximate correction for the latter, using the viscosity data for water of Svedberg & Pedersen (1940), reduces the mobilities of the chief components of fractions 0/33 and 40/50 at 20° from 0.38 and $0.93 \mu/\text{sec}/V/\text{cm}$ to 0.24 and $0.59 \mu/\text{sec}/V/\text{cm}$, at pH 7.5 in phosphate buffer, at 4° and these values are sufficiently close to the experimental results at 4° , at pH 7.0 in phosphate buffer, namely, 0.18 and $0.51 \mu/\text{sec}/V/\text{cm}$. Unfortunately it was not possible to extend the present work to include measurements at a series of different pH values at the two temperatures and so describe the electrophoretic properties of the fractions more completely.

Comparison of the present results at the appropriate temperature can be made with those obtained by other workers on proteins of bovine origin this is shown in Table 6, where the mobilities have been

rounded off to the first decimal place, and when necessary the average of the ascending and descending mobilities has been taken. The mobility of the chief component of fraction 0/33 at 4° does not correspond to that of any of the normal serum globulins, being about twice as fast as bovine γ globulin it is approximately the same as that found for the immune globulin of colostrum and whey, and the T-component of immune plasma by Smith (1946*a, b*), the component α_2 , of colostrum described by Gronwall (1947) and the γ globulin appearing in the sera of young calves on the ingestion of colostrum, reported by Jameson, Alvarez-Tostado & Sortor (1942) and San Clemente & Huddleson (1943).

The mobility of the main component of fraction 40/50 at 4° was similar to that of the main component of fraction A, casein, of colostrum, described by Smith (1946*a, b*) and to that of component α_1 , one of the characteristic proteins of colostrum found by Gronwall (1947). As the amount of casein in pre colostrum was very small and any present was removed in the fractionation process, the main component of this fraction is possibly the same as the protein α_1 , referred to above. The mobility of the fastest component of fraction 40/50 at 20° is similar to that found by Pedersen (1937) for lactoglobulin at this temperature.

The coloured component appearing in both fractions showed a mobility similar to that of other proteins described in colostrum, but as the colour of the latter has received no special comment, the resemblance is probably superficial.

Fraction 0/33, it has been noted, contained one main component. The diffuseness of the bands in the experiments at 4° , and the flatness of the curves in the experiments at 20° , appear greater than might be expected to arise from diffusion alone, and suggest that the preparation was heterogeneous. Unfortun

Table 6 *Comparison of mobility of precolostral proteins and of other bovine proteins*

Reference	Protein	Mobility* ($\mu/\text{sec}/V/\text{cm}$)	Temp ($^\circ$)	pH	Buffer
Present paper	Precolostrum, fraction 0/33	0.2	4	7.0	Phosphate
Smith (1946 <i>a, b</i>)	Colostrum, immune globulin	0.2	1	8.5	Borate
Smith (1946 <i>a, b</i>)	Whey, immune globulin	0.2	1	8.4	Veronal
Smith (1946 <i>a, b</i>)	Plasma, T component	0.2	1	8.6	Veronal
Smith (1946 <i>a, b</i>)	Plasma, γ globulin	0.1	1	8.6	Veronal
Gronwall (1947)	Colostrum, component α_2	0.2	—	7.7	Phosphate
Jameson <i>et al</i> (1942)	Serum of suckling calf, γ globulin	0.2	0.5	7.7	Phosphate saline
Present paper	Precolostrum fraction 40/50, chief component	0.5	4	7.0	Phosphate
Smith (1946 <i>a, b</i>)	Colostrum, fraction A, casein, chief component	0.5	1	8.4	Veronal
Gronwall (1947)	Colostrum, component, α_1	0.5	—	7.7	Phosphate
Present paper	Precolostrum, fraction 40/50, fastest component	1.5	20	7.5	Phosphate
Pedersen (1937)	Lactoglobulin	1.5	20	7.5	Phosphate

* These figures have been rounded off to the first decimal place

ately, the runs had already taken too long for it to be practicable to test this point by reversal of the current. However, the heterogeneity constant (H), defined by Sharp, Hebb, Taylor & Beard (1942), was assessed from the second moments of the gradient curves of Fig 1a and the values 0.047 and 0.054 μ /sec/V/cm obtained for the ascending and descending boundaries respectively, these values would be slightly reduced by allowing for the effect of diffusion on a slow-moving boundary. They compare with the value found by Sharp *et al* (1942) for their horse pseudoglobulin I preparation, viz 0.07 μ /sec/V/cm. The heterogeneity was further tested by calculating the third and fourth moments of the curves of Fig 1a at the end of the run; these indicated that the mobilities were normally distributed.

The heterogeneity found might have arisen during the course of fractionation which involved ammonium sulphate precipitation and dialysis at room temperature and extended over some 3 weeks, but this cannot be asserted without comparable data for this component before fractionation, which were not obtained in the present investigation.

Further examination of fraction 0/33

Of the two fractions obtained by salting out, fraction 0/33 was found to contain an antibody globulin and to consist of one electrophoretic component. The properties and purity of this fraction were therefore further investigated. The electrophoretic behaviour of the adsorbed protein was studied to give the iso-ionic point of the protein and to allow a comparison of the mobilities of the dissolved and adsorbed protein; the mean molecular weight was studied by osmotic pressure measurements, the number of thermodynamic components was investigated by solubility methods and the nitrogen content and the partial specific volume determined.

Electrophoresis of the adsorbed protein. The mobility of the protein adsorbed on droplets of Nujol (liquid paraffin) in buffers of varying pH and ionic strength was determined in a micro electrophoresis cell in an ultramicroscope. The emulsion was made by running a boiling solution of 1 ml Nujol in 20 ml ethanol through a fine capillary tube into boiling water (1 l), and removing the ethanol by boiling off about half the water. Acetate and phosphate buffers were made to cover eight pH values from 4.0 to 7.7, each at five different ionic strengths from 0.02 to 0.2, with the aid of a Cartesian nomogram based on the data of Green (1933). The solution for the mobility measurements was made by adding 1 ml of 1.5 g/100 ml solution of the protein in saline to 1 ml of Nujol emulsion in a 50 ml flask, adding the appropriate amounts of standard acetic acid and NaOH or KH_2PO_4 and KOH solutions, and making up to a volume with water. The solution after standing 15 min was introduced into a double-tubed cell similar to that of Smith & Lisse (1936), but fitted with reversible Ag/AgCl electrodes in compartments separ-

ated from the rest of the cell by a constriction plugged with cotton wool. The electrodes were connected through a milliammeter and a reversing switch to a variable resistance connected across 200 V d.c. mains. A voltmeter could be connected across the electrodes when desired. The applied potential was varied so that the current flowing through the cell did not exceed 5 ma, in order to avoid any convection disturbances through the heating effect of the current. The velocity of the adsorbed protein was obtained by timing ten droplets in both directions across a calibrated eye piece micrometer, with a stopwatch reading to 0.1 sec. These measurements were made at the central stationary level of the smaller tube, this level was easily determined experimentally by a series of measurements at various apparent depths. The conductivity and pH of the solution were measured after its removal from the cell.

It was difficult to maintain constant conditions of measurement in some cases because of the heat produced by the lamp. Some typical figures in Table 7 show that the temperature

Table 7 *Constancy of conditions of measurement of mobility in the micro electrophoresis cell*

	Potential difference (V)					
	88	88	84	108	110	125
Start	88	86	84	110	110	127
Finish						
	Current (ma)					
	4.3	4.28	4.0	3.97	4.2	4.5
Start	4.5	4.68	4.28	4.18	4.35	4.95
Finish						
	Temp (°)					
	23	23	24	26	27.5	25
Start	24	25	25.5	27	28	25.5
Finish						

and (probably as a result of this) the current flowing increased, while the applied potential remained constant. Consequently, it was more convenient to calculate the potential gradient from the potential difference across, rather than from the current through, the cell. Any error involved in this procedure was small since good agreement was obtained with figures based on a mid value for the current, as shown in Table 8.

Table 8 *Comparison of the values of the potential gradient obtained from (a) the potential difference and the length of the capillary tube, and (b) the current and the conductivity of the solution*

Method of calculation	Potential gradient (V/cm)							
	12.8	12.8	22.5	22.5	8.8	8.8	6.3	6.3
(a)	12.2	12.0	23.4	22.5	8.7	8.8	6.1	6.0
(b)								

The final protein concentration obtained in this method namely 0.03 g/100 ml, was shown to be sufficient to cover the droplets by (1) determining the pH mobility curve of Nujol droplets alone at 0.1 ionic strength, which showed mobilities several times greater than when protein was present, and (2) determining the mobility of droplets at pH 7.48 and ionic strength 0.1 in the presence of a decreasing concentration of protein, at the lowest protein concentration, 0.001 g/100 ml, this showed droplets of two distinct mobilities, indicating coated and uncoated droplets, and at higher protein

concentrations, a uniform value, decreasing slightly with increase of protein concentration, probably because of the effect of viscosity

The pH mobility curves were drawn from the data obtained at different ionic strengths (not included here). The appropriate mobility of the adsorbed protein, $0.97 \mu/\text{sec}/V/\text{cm}$, was considerably higher than that, $0.4 \mu/\text{sec}/V/\text{cm}$, found for the dissolved protein in the Tiselius apparatus at 20° , but was close to the value, $0.93 \mu/\text{sec}/V/\text{cm}$, found for the chief component of fraction 40/50 in solution

The iso ionic point of the adsorbed protein was derived from the pH mobility curves as follows: (1) the mobility at different ionic strengths for each of eight constant pH values was interpolated and plotted against the square root of the ionic strength, giving lines (Fig 2) that were extrapolated to

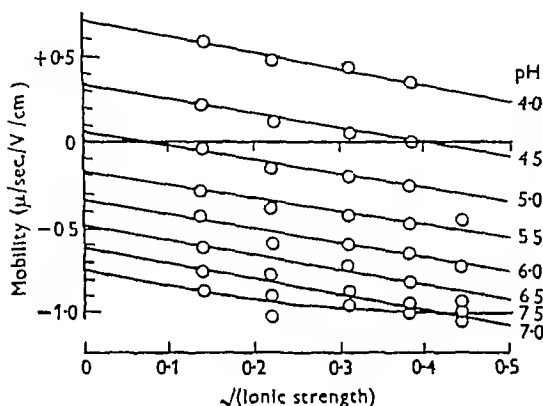


Fig 2

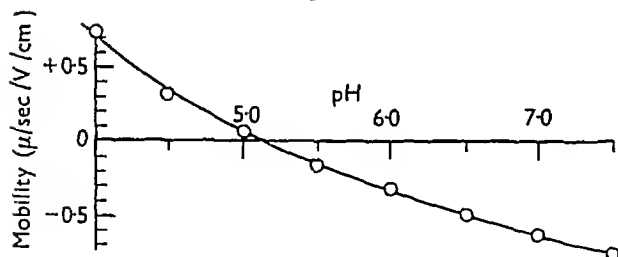


Fig 3

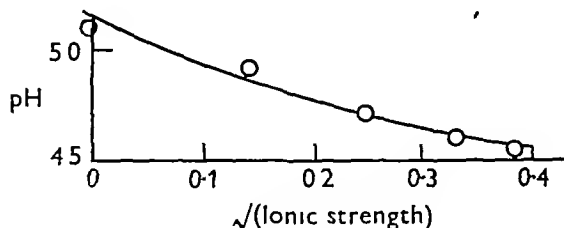


Fig 4

give the pH mobility data at zero ionic strength (Fig 3) from which an iso ionic point of pH 5.13 was obtained, (2) the iso electric points at the different ionic strengths were interpolated and plotted against the square root of the ionic strength (Fig 4) and extrapolated to give an iso ionic point of pH 5.15

The different mobilities of the protein in the adsorbed and dissolved state may be explained by increased ionization on adsorption. There are, how-

ever, few proteins for which data are available for making a comparison like this. Horse serum albumin, egg albumin and horse serum pseudo globulin were studied in solution by Tiselius (1930, 1937*a, b*) and adsorbed on inert particles by Abrahamson (1932) and Moyer (1938*a, b*). The data on serum albumin showed good agreement, but in the case of egg albumin, the pH-mobility curve of the adsorbed protein was slightly displaced to give an isoelectric point at pH 4.82 instead of at pH 4.55. The data for adsorbed pseudoglobulin showed good agreement with the early work of Tiselius in which the migration of the boundary was followed by ultra violet adsorption at 20° . This agreement, however, requires qualification as Tiselius later resolved the pseudoglobulin into α - and γ globulin with his improved apparatus working at 0° , so that this comparison has not been made on a pure protein. Adsorbed unfractionated horse serum globulin was studied by Moyer & Moyer (1940), and was found to behave like the α -globulin of Tiselius, if allowance was made for the different temperatures of measurement, this suggested that adsorption had been selective. There is thus some evidence against the view that adsorption affects the ionization of a protein. This favours an alternative explanation of the present results, viz. that a trace of some substance more surface-active than the chief constituent of the fraction was present. This possibility is supported by similar mobilities noted for the adsorbed protein and the chief component of fraction 40/50 in solution.

Mean molecular weight. The osmotic pressure of a series of dilutions of the fraction in saline was determined in an Adair type osmometer in a thermostat at 20° . The position of the descending inner meniscus was followed with a cathetometer, the initial rapid fall of the meniscus was succeeded by a slight but steady decrease due to slow denaturation of the protein. The equilibrium position was obtained by plotting the observations and extrapolating to zero time. At the end of the experiment, the osmometer was dismantled and the capillary correction, pH and nitrogen content of the solution determined. The corrected pressure p was assumed to be due to protein of concentration c , calculated from the nitrogen content and an experimentally determined factor 6.60 (see p. 540) as the Donnan effect of the protein in normal saline should be negligible.

The results are shown in the graph of p against c in Fig 5. Except at the lowest concentrations, the points fall on a straight line which, however, does not pass through the origin. The molecular weight was evaluated by plotting p/c against c in three ways, shown in Fig 6: (1) line A corresponds to the experimental data and indicates an uncorrected molecular weight of 170,000 at the higher concentrations, but is too curved to extrapolate to $c=0$; (2) line B is derived from the curve drawn to fit the experimental data and to pass through the origin, on extrapolation to $c=0$ it gives a molecular weight of 300,000; (3) line C is obtained from the experimental data adjusted to an origin at the intersection of the straight line through them with the c axis, on extrapolation to $c=0$ it gives a molecular weight of 146,000.

As the results obtained at the lowest concentrations are less accurate, because of the relatively greater capillary correction, than those obtained at higher concentrations, the latter, which fell approximately on a straight line, are more

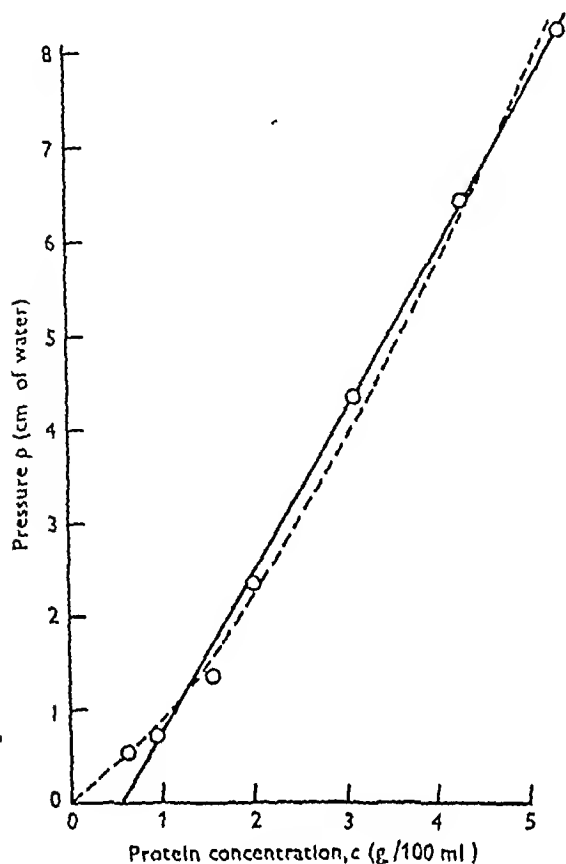


Fig 5

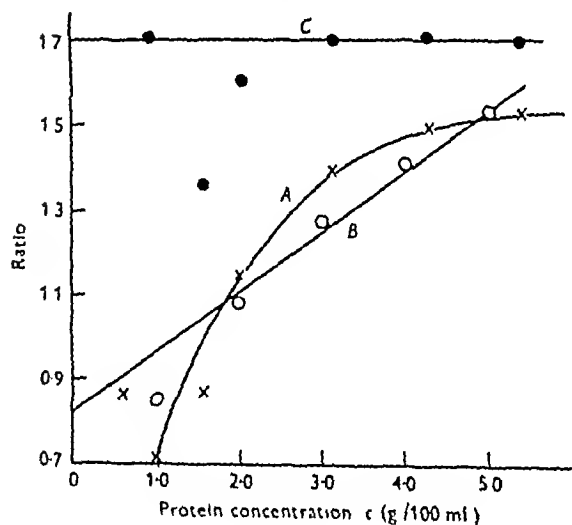


Fig 6

significant and favour treatment (3) above. The failure of the line, however, to pass through the origin is difficult to interpret, it clearly cannot be due to any effect that is a function of the protein concentration. It may indicate that a constant amount of protein was denatured on setting up each osmometer or that the membranes had an asymmetry

potential giving anomalous osmosis. As there is no evident reason why either process should have occurred, it is probably more plausible to accept tentatively treatment (2) giving line B and an apparent molecular weight of 300,000.

This value is somewhat higher than those found for immune bovine colostrum globulins by Smith (1946a, b), 180,000, and for serum globulins by Adair & Robinson (1930), 175,000, and by Burk (1937), 173,000. It is quite different from that found for lactoglobulin by Pedersen (1937), 41,500 and 38,000 and for the immune globulin of bovine anti pneumococcus serum by Kabat (1939), 910,000.

Solubility As the purity of a preparation of high molecular weight is not established by any one criterion such as electrophoretic mobility, the number of thermodynamic components in the fraction was investigated by two solubility methods, namely, the salting out curve of Colm (1925)—solubility as a function of salt concentrations—and the phase test—solubility as a function of total amount of solute.

The salting out curve The protein solution (0.5 ml) was added to 15 ml portions of a series of dilutions of a 3M phosphate buffer at pH 6.5, prepared according to Butler & Montgomery (1932), left standing in a water bath at 20° overnight, filtered through a no. 50 Whatman paper and the nitrogen determined on 2 ml of the filtrate. The solubility is plotted on a logarithmic scale against the molarity of the phosphate buffer in Fig 7. It is evident that the protein has been precipitated in two stages between the limits 1.2–1.4 and 1.4–2.1 M, the salting out constants, K_s' , being 0.22 and 0.01 respectively.

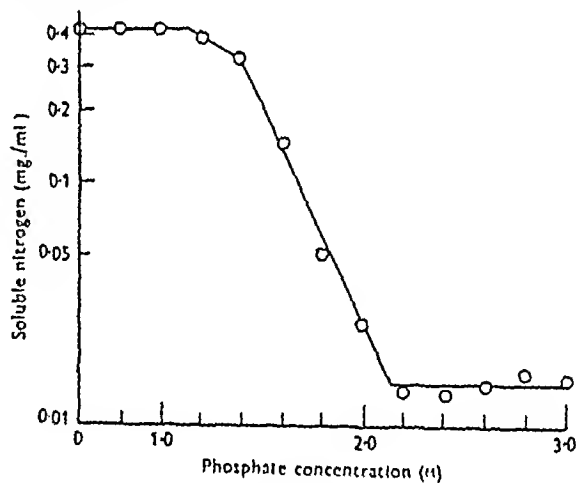


Fig 7

The phase test Increasing amounts of the protein solution were made up to 15 ml with distilled water and phosphate buffer to give a final phosphate concentration of 1.1 M at pH 6.5, the solutions were then shaken vigorously and left standing in a water bath at 20° overnight, filtered through a no. 50 Whatman paper and the nitrogen determined on 2 ml of the filtrate. The total N calculated from the volume and concentration of the protein solution used, and the soluble N found are given in Fig 8. This shows three parts to the solubility curve, indicating saturation with components at A and B and the existence of at least a third component whose point of saturation was not attained. The

solubilities of the first two components were obtained from the intercepts of the extrapolated portions of the curve on the ordinate A' and B' and found to be, in terms of N, 0.06 and 0.27 mg/ml respectively. The proportions determined graphically by the procedure shown in Fig. 9 were found to be 53 and 17% of the protein respectively.

is given in Table 9, which shows an average value of 15.15%, equivalent to a factor of 6.60. This value is a little lower than that reported by Smith (1946*a, b*) for the immune globulin of bovine colostrum, namely 15.44 and 15.53%, but is close to the value of 15.2% found for serum globulin by Adair & Robinson (1930). The partial specific volume was found to be

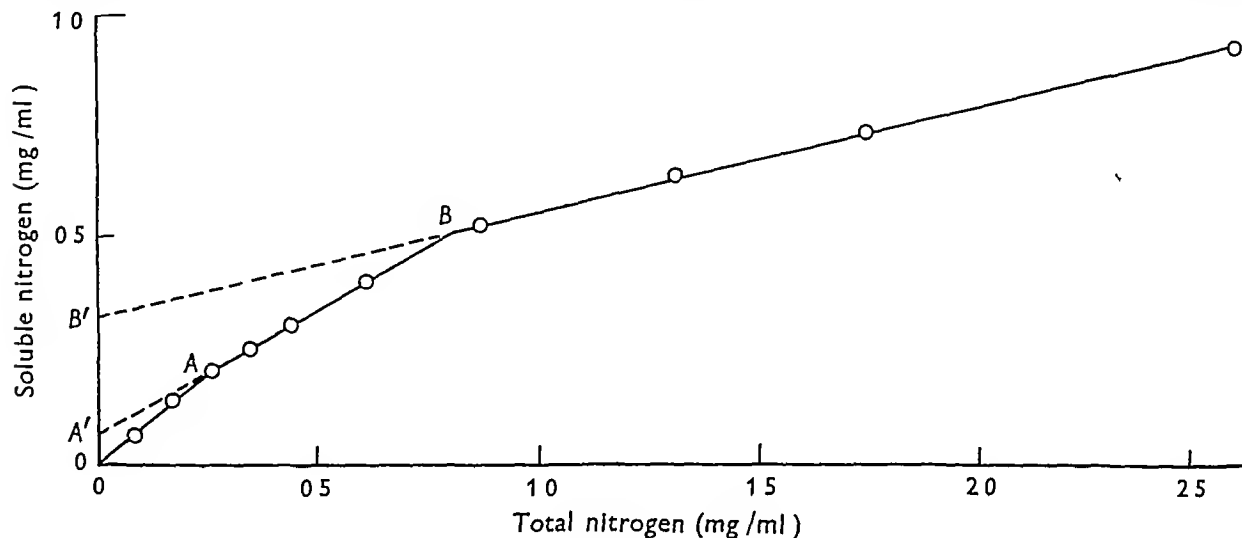


Fig. 8

It is clear from both these tests that the fraction contained several components, although electrophoresis had revealed only one. Comparison of the salting out curve with the findings of Butler & Montgomery (1932) that the euglobulin and pseudoglobulin of human and of horse serum precipitated

0.723, a value close to the average of 0.75 for serum globulin, which suggests that the present fraction, like the γ globulin of serum, is not associated with any appreciable quantity of lipids.

Table 9 Nitrogen content of protein fraction 0/33

N (g/100 ml. solution)	N (% of ash free protein)
0.473	15.12
0.815	15.30
1.34	15.13
1.34	15.11
1.34	15.13
1.34	15.09
Average	15.15
N factor (=100/15.15)	6.60

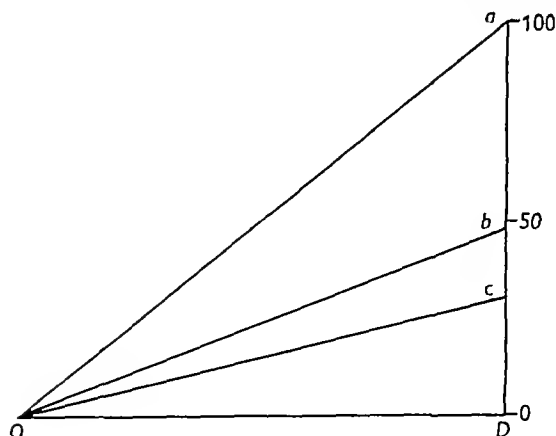


Fig. 9

between the limits 1.2–1.6M and 1.5–2.5M, and the finding of Butler, Blatt & Southgate (1935) that horse pseudoglobulin was salted out with $K'_s = 0.5$ in the same buffer system, suggests that the proteins in the present fraction are probably distinct from the main serum globulins.

Nitrogen content and partial specific volume. The N content and partial specific volume were determined on saline solutions of the fraction by drying to constant weight at 110°, and by pycnometry at 20° respectively. The N content

DISCUSSION

The extent to which an immune globulin fraction can be regarded as a distinct entity is difficult to decide. Biological criteria show that many different antibodies may be chemically indistinguishable, and that the same antibody may have different chemical properties, depending on the species in which it is formed, the manner in which its formation is stimulated and the stage in the response to the antigenic stimulus. In the present work, tests were made for the *Brucella* agglutinin only, and this was found to be confined to fraction 0/33 of the salting out fractions. As other antibodies of the secretion may have similar solubilities, this fraction is probably not biologically pure and may be described tentatively as the immune globulin of bovine precolostrum. The extent to which this fraction is chemically homogeneous may be judged from the present results in

several ways. Electrophoresis of the dissolved protein showed one component probably accounting for all the fraction. Electrophoresis of the adsorbed fraction suggested that a minor component may have been preferentially adsorbed. The salting-out curve showed a chief component and one other, and the phase test a chief component and two others. In considering these results it should be borne in mind that electrophoresis of the adsorbed protein may afford a very sensitive qualitative test of the purity of the protein, as it would be affected by the presence of quite small amounts of other proteins if these were more surface active than the main constituent, and that the phase test is one of the most sensitive tests of purity available, since a protein may appear homogeneous in the ultracentrifuge and Tiselius apparatus and still fail this test. It is therefore considered that while the present fraction is not pure, it does represent a promisingly high degree of separation of the main component of precolostrum with which the immune properties of the latter appear to be associated.

The properties of an immune globulin from bovine precolostrum are not without significance in connexion with the formation of this material and its relation to the subsequent secretions from the bovine udder. This aspect of the present results and of other experiments will be discussed elsewhere by Blakemore & McDougall (unpublished). Here it may be pointed out that the resemblance noted between the main electrophoretic component of precolostrum and the T component of bovine plasma suggests that the precolostrum globulins are secreted from the blood stream, a view which is supported by the antibody studies of Blakemore (1947). The globulins of precolostrum resemble those of colostrum, in that a high proportion of them are easily salted out, that the easily salted out globulins bear the *Brucella* agglu-

tinins and probably other antibodies of the secretion, and that the main globulin fraction resembles the immune globulin of colostrum, in the mobility of its main electrophoretic component, its approximate molecular weight, and its salting-out limits. These similarities support the view on the interrelation of precolostrum, colostrum and milk mentioned in the introduction, and suggest that an adequate dry period is necessary before calving to yield the best colostrum.

SUMMARY

1 Bovine precolostrum globulins contain a high proportion of Howe's (1922) euglobulin and pseudo-globulin I fractions, i.e. those most easily salted out.

2 An easily salted-out immune globulin fraction has been prepared by ammonium sulphate precipitation at 33% saturation.

3 This fraction contained one electrophoretic component with a mobility of $0.2 \mu/\text{sec}/\sqrt{V}/\text{cm}$ at 4° , in phosphate buffer at pH 7.0 and ionic strength 0.1, but solubility tests in phosphate buffers indicated more than one component. It had an apparent molecular weight (tentatively proposed) of 300,000, a nitrogen content of 15.15% and a partial specific volume of 0.723.

4 The properties of this fraction are discussed in relation to the T-component of bovine plasma and the immune globulin of bovine colostrum.

I am indebted to Mr F. Blakemore, Veterinary Investigation Officer, Institute of Animal Pathology, Cambridge, for the supply of material and for the examination of the fractions for antibody, to Prof E. K. Rideal and Dr P. Johnson, Colloid Science Department, Cambridge, for facilities for and encouragement in much of the physico-chemical work in the present investigations, and to Dr E. C. Bate Smith, Superintendent, Low Temperature Research Station, Cambridge, for facilities for some of the electrophoresis measurements.

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A Further Study of Hydrolysates of Gramicidin

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Most of the following experiments had been done before the author learnt that Gregory & Craig (1948) had shown that gramicidin, as previously prepared and studied, was a mixture of substances. This work seems, nevertheless, to be worth reporting, since a statement of technical successes and failures and of results obtained may prove helpful for future studies of individual gramicidins. Furthermore, evidence was secured which was very difficult to reconcile with the assumption that gramicidin is homogeneous, and which may throw some light on the nature of the differences between individual gramicidins.

The present work is a continuation of work with acid hydrolysates of gramicidin previously published (Gordon, Martin & Syngé, 1943, Syngé, 1944, 1945*a, b*). The main object was to ascertain the numbers, sequence and optical configuration of the different amino-acid residues in the molecule.

Amino-acid composition: slow liberation of valine in acid hydrolysis

In the first place, acid hydrolysates prepared at high temperatures were analyzed for amino acids by the procedure of Gordon *et al.* (1943) and with closely similar results (see Table 2, p. 545). In all, five different preparations of gramicidin were compared, and no definite differences in amino acid composition were observed. One of the specimens (13941) crystallized readily from aqueous ethanol, giving chunky, well-formed crystals. Of all specimens previously studied, only one had ever yielded such crystals, and it had proved impossible to reproduce the phenomenon. Crystalline and mother-liquor fractions obtained from 13941 by crystallization from aqueous ethanol did not differ significantly from the parent material in amino-acid composition or optical rotation. A counter-current extraction of 13941 carried out by Dr Craig confirmed, however, that the material was heterogeneous (though containing a proportion of gramicidin A rather higher than usual). Thus crystallization from aqueous ethanol is no more capable of effecting separation of the different gramicidins than the usual crystallization from acetone.

On comparing the amino-acid data for different conditions of hydrolysis, it is seen that consistently more valine is obtained with more vigorous hydrolysis (Table 2). Previous valine figures must therefore be

regarded as too low. Prolonging the period of hydrolysis to 48 hr. led to a marked diminution in the amount of the second of the two 'anomalous' bands noted in purifying the leucine fraction (cf. Gordon *et al.* 1943). It is reasonable to suppose that the last valine to be liberated is derived from the somewhat slow hydrolysis of valylvaline (Christensen 1943, 1944) and that the second 'anomalous' band is due to acetylvalylvaline. Dr Gregory (personal communication) states that he, too, has isolated valylvaline after vigorous acid hydrolysis of gramicidin. He also states that phenylalanine is present as a component of gramicidin B, it is presumably a small amount of acetylphenylalanine from this source that is responsible for the first 'anomalous' band. The finding of substantial amounts of unhydrolyzed peptides after performing hydrolysis under such conditions emphasizes the importance, in amino acid analysis of proteins, of demonstrating rather than assuming completeness of hydrolysis. The revision upwards of the figure for the valine content of gramicidin leads to a satisfactory account for its entire carbon content, and abolishes the need for postulating hitherto unrecognized residues (cf. Syngé, 1945*b*).

The valine isolated after hydrolysis with hydrochloric acid at 110° for 48 hr. was found to be racemic, whereas with hydrolysis for 10 days at 37° followed by 48 hr. at 110° there was a marked preponderance of D-valine, as in previous work (Syngé, 1944).

Further study of partial acid hydrolysate: identification of dipeptides

The faster running fractions from the starch *n*-butanol-water chromatogram of a 10 day acid hydrolysate of gramicidin (Syngé, 1944) were subjected to further qualitative study by published methods (Consden, Gordon & Martin, 1944, 1947, Sanger, 1945, Porter & Sanger, 1948). It was established that, in addition to the valylglycine (fractions 26-31) found previously (Syngé, 1944), fractions 21-19 were a mixture of free valine, leucylglycine and alanylvaline (and not, as had previously been suggested on inadequate grounds, a tripeptide of leucine, valine and glycine). Fractions 18-14 contained free tryptophan and free leucine, fractions 13-11 alanylleucine and fraction 10 material apparently chromatographically homogeneous with all solvent systems tried, this last fraction could have been

alanylvalylleucine, alanylleucylvaline or a mixture of these. The three newly recognized dipeptides were further identified by comparison on mixed chromatograms with synthetic D-leucylglycine, L-alanyl-D-valine and L-alanyl-D-leucine. The synthesis of the two latter compounds is described in the Experimental section.

Table 1 shows the sequence of the different peptides and amino-acids on emerging from the original starch chromatogram. It should be remembered that the substances fractionated were originally in the form of their hydrochlorides, which may run in a different sequence from free amino acids and peptides (Synge, 1944, Moore & Stein, 1948).

Table 1 *Products of partial hydrolysis of gramicidin so far identified*

(The compounds are listed in the sequence in which they emerged from the original *n*-butanol-water starch chromatogram (Synge, 1944). Fraction numbers and *R_f* values refer to this chromatogram.)

Fraction no	Approx <i>R_f</i> value	Compound	Amount (N of compound isolated as % of total N of gramicidin)	Reference
37-40	0.10	Alanine	2.5	Synge (1944)
25-31	0.3	L-Valylglycine*	Approx. 7	"
17-22	0.5	Valine	0.45	Present work (see also Synge, 1944)
17-22	0.5	L-Alanyl-D-valine*	0.45	" "
17-22	0.5	D-Leucylglycine*	1.0	" "
14-18	0.6	Tryptophan	—	" "
12-16	0.75	Leucine	—	" "
11-13	0.9	L-Alanyl-D-leucine*	Little	" "
9-11	1.1	?(Alanylvalylleucine or alanylleucylvaline)	Little	" "
4-10	1.4	?(Dipeptides of higher amino acids, perhaps including L-valyl-L-valine* and D-valyl-D-valine*)	Much 2.6 2.6	" Christensen (1944) "
1-4	2.0	?(Tripeptides, etc. of higher amino acids)	Much	Present work

† See Martin & Synge (1941)

* These peptides have been identified conclusively

Attempted fractionation of peptides present in faster running fractions

It did not prove easy to identify or isolate individual peptides from fractions faster than fraction 10. Repeated attempts were made at further fractionation on paper chromatograms, but the materials either ran very fast in the more polar solvents such as phenol and collidine or else tailed very badly or did not run at all in the less polar solvents and solvent mixtures used (light petroleum, carbon tetrachloride, etc.). Some of the materials also gave very poor colours with ninhydrin. This last behaviour is typical of higher peptides, but also occurs with tryptophyltryptophan (Synge & Tiselius, 1949). An impression was gained by hydrolysis of some of the fractions, and by comparison with synthetic model substances, that the ninhydrin and Ehrlich peak around fraction 6 was primarily due to dipeptides such as leucylleucine, leucyltryptophan, etc., while that around fractions 1 and 2 was due to higher

peptides (tripeptides, etc.) of leucine, tryptophan and valine.

It is clear that mixtures of peptides of this kind (which represent by far the greater part of the hydrolysate) cannot be satisfactorily fractionated on ordinary silica, paper or starch partition chromatograms. Attempts, following the suggestion of Boscott (1947), to hold the less polar phase stationary with cellulose acetate as a supporting structure (either in the form of Celanese satin or of fully acetylated filter paper (cellulose triacetate)) gave disappointing results with simple amino-acids and peptides. Although it was clear from the relative rates of movement of the different substances that the less

polar phase was being held stationary in the chromatograms, excessive 'tailing' occurred, which may be attributed to adsorption effects on the cellulose acetate in the presence of such solvents as butanol and collidine. Perhaps better results may be obtained with rubber as a stationary phase as in the latex-impregnation technique of Bolding (1948).

Another promising approach to this problem is to use bulk countercurrent liquid extraction as used by Craig and his co-workers. This may largely eliminate undesirable effects due to adsorption, and permits working with partition coefficients more favourable to the less polar phase. The use of charcoal-adsorption methods may also facilitate separation of the peptides according to their tryptophan content, for which partition methods with the usual solvents are not very suitable (see Synge & Tiselius, 1949). Ionophoresis (Consden, Gordon & Martin, 1946) of the peptides, their acyl or *N*-2,4-dinitrophenyl derivatives in gels shows promise as a method for achieving fractionation according to ionic mobility,

which is an inverse function of molecular size. Thus leucine could be separated from leucylleucine or leucyltryptophan by ionophoresis of the dinitrophenyl derivatives in silica gel containing 0.2M-sodium acetate buffer at pH 6. The dinitrophenyl-peptides moved towards the anode at about half the rate of the dinitrophenylleucine. The true difference in ionic mobility is not of course so great since the substances were migrating against the electro-osmotic stream, and adsorption effects may also have contributed.

Optical form of amino acids from different peptide fractions: an ultramicro method for its determination

In view of the interest attaching to the optical form of the amino acids isolated after acid hydrolysis of gramicidin and other bacterial substances, it seemed important to ascertain the configuration after hydrolyzing further the individual partial hydrolysis products.

A semi-quantitative micro method was developed based on the specificity of the D-amino acid oxidase of kidney. Spots of an individual amino-acid, after separation on a paper chromatogram from other components of the mixture, were sprayed with a solution of the purified enzyme, and the extent of the attack on the amino-acid was gauged by comparing the spot with a number of control spots of optical antipodes of the same amino-acid mixed in varying proportions. The method requires as little as 30 μ g of the amino acid under examination. Jones (1948) has mentioned its use in a study of the hydrolysis products of 'aerosporin' (polymyxin). In the present work it has given satisfactory results with leucine and valine. It is not suitable for detecting small proportions of D-isomer mixed with excess of L-isomer, for which purpose L-amino-acid oxidases may in future prove useful. Obviously, the spraying of paper chromatograms with enzyme solutions is capable of innumerable other applications.

The experiments on the complete acid hydrolysates of the leucylglycine, alanylvaline and alanylleucine isolated as described above indicated, as expected, that the leucine from the leucylglycine and alanylleucine was largely the D-form. The valine from the alanylvaline proved to be predominantly the D-isomer. All these products were, however, to a considerable extent racemic (see Table 4). It was confirmed by the new method that the valine from the valylglycine (Synge, 1944) was predominantly the L-form. Alanine appeared to be predominantly the L-isomer.

Thus different partial hydrolysis products have been isolated yielding respectively an excess of L-valine (L-valylglycine by Synge, 1944, and L-valyl-L-valine by Christensen, 1944) and of D-valine (L-alanyl-D-valine in the present work and D-valyl-D-valine by Christensen, 1944). If one starts from

conventional premises it may be argued that valine residues of each of the two antipodal forms exist preformed in one or more of the molecular species constituting 'gramicidin'. However, a more cautious interpretation seems advisable in view of the fact that most of the products, whose optical character has been determined so far, are partly racemic. As previously implied in a footnote (Synge, 1944) and more explicitly stated by Neuberger (1948) there is no reason why a process similar to that which induces simple racemization in a free amino-acid in the presence of mineral acid should not, when the same residue still exists in peptide linkage with other optically active amino acid residues, cause epimerization or inversion of the residue in question. It is not justifiable, therefore, in the present state of our knowledge to deduce rigorously from the optical configuration of free amino acids found in hydrolysates the configuration of the corresponding residues in the intact molecule. Extensive model experiments with optically active peptides are required.

Conclusion

When it appeared reasonable to suppose that gramicidin was a single homogeneous substance, it was argued that, since the molecule could not contain more than two glycine residues, and since more than half the total glycine had actually been isolated as valylglycine, all the glycine residues must occur linked to valine through their amino groups (Synge, 1944). This view became untenable when leucylglycine was found to occur in the same partial hydrolysate with valylglycine. Gregory & Craig's (1948) demonstration of the heterogeneity of gramicidin resolves this difficulty. It would be the part of wisdom to expect similar anomalies in studies of partial hydrolysis of 'pure' proteins as evidence accumulates that calls for scepticism as to their homogeneity.

At the present time it is impossible to put forward any extensive hypothesis as to the structure of individual species of gramicidin on the basis of partial hydrolysis studies made with the mixture. All that can be done is to list the peptides so far identified in partial hydrolysates of mixed material, with their probable optical configurations. This is done in Table 1, where those peptides that have been conclusively identified are marked with an asterisk. Future studies of partial hydrolysates of individual gramicidins will be required for further progress towards determining sequences of amino-acid residues.

EXPERIMENTAL

Melting points are uncorrected. Optical rotations were determined in a 0.5 dm tube. Carbon and hydrogen determinations were by Drs Weiler and Strauss, Oxford.

Materials

Gramicidin specimens W_2 and R_3 (Syngé, 1944, 1945a, b, Syngé & Tiselius, 1947) were studied together with a batch of acetone recrystallized gramicidin (no 13941) kindly supplied by the Wallerstein Company, New York, during 1945. As already mentioned, this material differed from previously studied preparations in crystallizing fairly readily from aqueous ethanol on allowing slow evaporation of the solution. Specimens of the resulting crystals (13941 X) and mother liquor (13941 ML) were separated after about half of the material had crystallized. Optical rotations (c , 1.5 in 94.5% (w/v) aqueous ethanol) were as follows: W_2 , $[\alpha]_D^{25} +3^\circ$, R_3 , $[\alpha]_D^{27} +5^\circ$, 13941, $[\alpha]_D^{23} +6^\circ$, 13941 X, $[\alpha]_D^{24} +4^\circ$, 13941 ML, $[\alpha]_D^{24} +2^\circ$ (calculated for materials dried to constant wt in a vacuum desiccator over H_2SO_4 and soda lime at room temperature).

The fractions obtained (Syngé, 1944) by starch partition chromatography (*n* butanol water) from a hydrolysate of gramicidin R_3 were further studied. Hydrolysis had been in equal volumes of 10N HCl and glacial acetic acid for 10 days at 37° . Fractions are numbered as in the original paper.

Optical rotation of valine specimens

Acetylvaline, isolated by partition chromatography as above from hydrolysates of gramicidin (13941), was hydrolyzed with 6N HCl for 24 hr at 110° . The product was evaporated to dryness and weighed. From the weight of valine HCl found, the weight of valine present was calculated. The optical rotation of the product was determined. Results are shown in Table 3.

Table 3 *Optical rotation of valine isolated from gramicidin after hydrolysis*

(Hydrolysis in acetic acid + 3.5 vol of 6N HCl $[\alpha]_D$ measured in 6N HCl)

Duration and temperature of hydrolysis	$[\alpha]_D$ ($^\circ$)	Temp ($^\circ$)	c
24 hr at 110°	-1	19	0.7
48 hr at 110°	± 0	19	1.7
10 days at 37° followed by 48 hr at 110°	-6	17	1.2

*Study of fractions obtained from *n* butanol water starch partition chromatogram of partial hydrolysate (Syngé, 1944)*

In general, small portions of these fractions were subjected to further study on paper chromatograms. Amino acids and peptides were made visible by spraying with ninhydrin, tryptophan compounds by Ehrlich's reagent (cf Syngé & Tiselius, 1949). In addition, observation of the chromatograms in ultraviolet light showed the presence of a variety of intensely fluorescent substances that did not colour either with ninhydrin or Ehrlich's reagent. They 'tailed' considerably and amino acids and peptides either displaced them or quenched their fluorescence. These fluorescent spots thus served as markers for cutting the chromatograms. They were thought to be breakdown products of tryptophan. After hydrolysis of peptide material, constituent amino acids were detected routinely on *n* butanol H_2O one dimensional chromatograms with Munkell OB paper. All five amino acids could readily be identified in this way, tryptophan falling conveniently between leucine and valine.

Table 2 *Amino acid analyses of gramicidin hydrolysates, with varying hydrolysis conditions*

(Amino acid figures are N as % of total N of gramicidin (Kjeldahl-Friedrich))

Hydrolysis mixture	Syngé (1944)		Gordon <i>et al</i> (1943)		Present work													
	0.8 ml acetic acid 3.0 ml 5N-HCl		0.8 ml acetic acid, 3.0 ml 6N-HCl		0.8 ml acetic acid, 3.0 ml 6N HCl													
	100°		110°		110°													
Temperature	100°		110°		110°													
Duration (hr)	48		24		24				48									
Specimen	R_3	W_2	Various		R_3	R_3	W_2	W_3	R_3	R_3	W_2	W_2	13941	13941	13941	13941	13941	13941 ML
Leucine N	23.5*	25.2*	20.0-21.6		20.0	21.0	20.0	20.3	19.8	20.4	20.8	20.6	19.6	10.7	20.3	20.3	20.4	20.6
Valine N	13.9	15.7	15.6-17.0		15.9	16.4	16.9	15.8	18.3	18.1	18.7	18.6	17.9	18.3	18.4	18.5	18.9	17.3
Tryptophan N	38.4	41.1	33.5-44.8		37.5	38.0	40.0	40.5	32.8	35.1	40.5	39.7	35.4	36.3	36.6	35.7	31.6	33.6
Alanine N	10.1	11.7	9.8-11.5		10.4	9.9	10.4	10.3	10.0	10.1	10.4	10.4	9.9	9.8	9.7	10.0	10.0	10.2
Glycine N	7.4	7.8	5.0-8.0		5.5	5.5	5.6	5.1	5.5	5.2	5.3	4.5	5.2	5.2	5.3	5.4	6.0	6.5

* Before chromatography with chloroform

Amino-acid analysis of gramicidin specimens

Specimens were hydrolyzed in hot HCl acetic acid, acetylated and analyzed for component amino acids by partition chromatography on silica gel exactly as described by Gordon *et al* (1943). The results are shown in Table 2 corrected (as previously) by the recovery factors for a known mixture of acetamido acids, subjected to exactly the same procedures and reagents. As before, the same anomalous bands were noted in the $CHCl_3$ chromatography of the leucine fraction. In the 48 hr hydrolysates, the anomalous band running behind the leucine band had become very much fainter.

Fractions 21-19 These fractions had already been pooled (Syngé, 1944) and were known to yield valine, leucine and glycine, with smaller amounts of alanine on complete acid hydrolysis. The material in the pooled fractions seemed best further fractionated with collidine. Paper chromatography with this solvent revealed free valine and, ahead of it, two spots very close to one another. These were so close that it was not easy to cut them apart. Accordingly, the two compounds were isolated together by running several one dimensional chromatograms in parallel, and eluting them with water after cutting away from free valine, etc. The extract was subjected to hydrolysis, with and without

previous deamination by nitrous fumes according to Consden *et al* (1947) The hydrolysate showed leucine and glycine as main and alanine and valine as subsidiary components Deamination abolished the leucine and alanine This suggested that the major component of the mixture was leucylglycine and the minor component alanylvaline The identification of the residues bearing the free amino groups was confirmed by treatment of the material with 1,2,4-fluorodinitrobenzene according to Sanger's (1945) techniques After acid hydrolysis *N* 2,4-dinitrophenylleucine and *N* 2,4-dinitrophenylalanine were identified qualitatively by mixed chromatography on narrow silica gel columns Finally, the identity of the peptides was confirmed by running mixed paper chromatograms with synthetic *D* leucylglycine (Hofmann-La Roche) and *L* alanyl-*D* valine (see below) with *n* butanol and *s* collidine as solvents At the same time it was established that in collidine leucylglycine is the faster moving of the two peptides From the amino acid analysis of the material in fractions 19–21 (Synge, 1944) it is reasonable to deduce that the free valine embodies 0.45% of the N of the original gramicidin, the leucylglycine 1.0% and the alanylvaline 0.45%

Fractions 18–14. Paper chromatography with *n* butanol, collidine, etc. of test portions of these fractions before and after acid hydrolysis showed the presence of free leucine and free tryptophan, the leucine predominating in the earlier fractions (14–16) and the tryptophan in the later fractions (17–18) There was also some overlap of alanyl leucine (see p. 547) into the earlier fractions and of leucylglycine into the later fractions No other components were recognized.

Fractions 13–11. Paper chromatography with *n* butanol (after neutralization with NH_3 vapour) showed a common constituent in all these fractions In fraction 13 it was contaminated with free leucine (which ran somewhat faster than the unknown compound) and in fraction 11 it was contaminated with the faster running peptide material also present in fraction 10 From Fraction 12, however, only a single spot resulted with a variety of solvents Fraction 12 was accordingly studied without further fractionation On acid hydrolysis, the material yielded alanine and leucine only With previous deamination, the alanine spot was weakened, though not abolished However, the fluorodinitrobenzene procedure yielded only dinitrophenylalanine and no dinitrophenylleucine Accordingly the material was compared by mixed paper chromatograms using both *n* butanol and *s* collidine with synthetic *L*-alanyl-*D* leucine (see p. 547) with which it appeared to be identical

Fraction 10. The material in this fraction gave only a single spot with ninhydrin on paper chromatography with a very wide variety of solvent mixtures On acid hydrolysis, it showed alanine, valine and leucine as spots of roughly equal intensity Previous deamination relatively weakened, but by no means abolished the alanine spot The fluorodinitrobenzene procedure yielded only dinitrophenylalanine Since the material could not be alanylleucine or alanylvaline, both of which, as hydrochlorides, had run more slowly on the original starch chromatogram and, as free peptides, on paper with *n* butanol, it is suggested that it may be alanylvalyl leucine or alanylleucylvaline or a mixture of both these peptides

Synthesis of L alanyl-D valine

Carbobenzyl-oxy *L* alanine was prepared (Bergmann & Zervas, 1932) from *L* alanine (made by resolution of DL

alanine according to Pope & Gibson (1912, cf. Fischer, 1899) Carbobenzyl-oxy *L* alanine (0.645 g) was allowed to react in 6 ml dry ether with 0.5 g PCl_5 at first at 0° and then at room temperature until all the PCl_5 had dissolved The resulting solution was washed quickly with ice water twice, and the ethereal solution of the acid chloride was then allowed to react for 2 hr at room temperature with 0.55 g *D* valine methyl ester in the presence of saturated aqueous KHCO_3 The ester (not characterized) had been prepared from *D* valine exactly as described by Synge (1948) for *L*-valine methyl ester The ether layer was then washed successively with dilute HCl and KHCO_3 and water, dried lightly over Na_2SO_4 , and on evaporation to dryness yielded a residue crystallizing readily from ether light petroleum Melting point and rotation were constant after one recrystallization (yield of recrystallized product, 0.3 g)

Carbobenzyl-oxy *L*-alanyl-*D* valine methyl ester had *m.p.* $89\text{--}90^\circ$, $[\alpha]_D^{21.5} +1^\circ$ (*c.* 1.4, ethanol) (Found C, 60.7, H, 7.2, N (Kjeldahl) 8.5 $\text{C}_{17}\text{H}_{23}\text{O}_5\text{N}_2$ requires C, 60.7, H, 7.1, N, 8.3%)

This compound was saponified by dissolving 0.27 g in 3 ml dioxan and adding 2 ml 1.2*N* NaOH The mixture was gently shaken until homogeneous, and kept for a further 0.5 hr at room temperature The mixture was then largely diluted with water, acidified with 0.5 ml 10*N* HCl (no precipitation occurred) and extracted with ether The residue from evaporation of the ether extract (carbobenzyl-oxy *L*-alanyl-*D* valine) could not be brought to crystallization, and was accordingly reduced with Pd H_2 in the usual way, after dissolving in a mixture of 10 ml methanol, 1 ml glacial acetic acid and 2 ml water After removal of Pd and repeated evaporation with water there was obtained a colourless, glassy residue (0.20 g) of *L*-alanyl-*D* valine The product would not crystallize from water-ethanol ether, nor on keeping in a desiccator, but crystallized slowly in rosettes (evidently with water of crystallization) on keeping in the laboratory air Paper chromatography indicated that the material was contaminated with a trace of free alanine, but was otherwise homogeneous R_F values, comparable with the data of Consden *et al* (1947), were, for *n* butanol water (with HCN) 0.21, and for *s* collidine water, 0.53 $[\alpha]_D^{19} +35^\circ$ (*c.* 2.8, water) (calculated, from *N* content of solution, for the anhydrous peptide) Air dry crystals were analyzed (Found C, 47.1, H, 8.8, N (Kjeldahl), 13.6, amino N (Van Slyke, 4 min) 52% of total N $\text{C}_8\text{H}_{16}\text{O}_3\text{N}_2 \cdot \text{H}_2\text{O}$ requires C, 46.6, H, 8.7, N, 13.6%, amino N 50% of total N)

Synthesis of L alanyl-D leucine

Carbobenzyl-oxy *L*-alanyl chloride prepared from 0.7 g carbobenzyl-oxy *L* alanine was allowed to react in 6 ml of washed ether solution in the presence of saturated aqueous KHCO_3 with *D* leucine methyl ester that had been liberated with K_2CO_3 in the usual way from 0.8 g of its hydrochloride (Smith & Brown, 1941) After 1.5 hr at room temperature the ether layer was separated, washed successively with dilute HCl and KHCO_3 solutions and with water, dried lightly over Na_2SO_4 and on evaporation yielded 0.87 g of syrup which could be crystallized from ether light petroleum The resulting carbobenzyl-oxy *L*-alanyl-*D* leucine methyl ester after two crystallizations (yield 0.71 g) had *m.p.* $71\text{--}73^\circ$, $[\alpha]_D^{20.5} +10^\circ$ (*c.* 1.6, ethanol) (Found C, 61.6, H, 7.5, N (Kjeldahl) 7.9 $\text{C}_{18}\text{H}_{26}\text{O}_5\text{N}_2$ requires C, 61.8, H, 7.4, N, 8.0%)

This compound was saponified by dissolving 0.37 g in 3 ml dioxan and adding 3 ml λ -NaOH. The mixture was kept for 0.5 hr at room temperature after it had become homogeneous. 30 ml water was then added, followed by 4 ml λ HCl. An oil precipitated which crystallized on keeping at 0°. Recrystallization to constant m.p. and rotation was effected from methanol/water. The product (0.22 g), characterized after drying in a vacuum desiccator at room temperature, appeared to be a hydrate of *carboxybenzyloxy L-alanyl D-leucine* m.p. 57–59°, $[\alpha]_D^{25} \pm 0^\circ$ (c, 1.0, methanol) (Found C, 58.1, H, 7.4, N (Kjeldahl), 7.0%, acid equiv. wt, 348. $C_{17}H_{23}O_5N_2 \cdot H_2O$ requires C, 57.7, H, 7.4, N, 7.9%, acid equiv. wt 354).

This material (0.17 g) was reduced with Pd/H₂ as above. The crystallization behaviour of the resulting glassy *L-alanyl D-leucine* was similar to that of *L-alanyl D-valine*, the peptide crystallizing over aqueous acetic acid, but not in the laboratory air nor in the desiccator. The material appeared homogeneous by partition chromatography on paper. R_F values comparable with those given by Consden *et al.* (1947) were, for *n*-butanol/water (with HCN), 0.33 and for *s*-collidine/water 0.60, $[\alpha]_D^{25} + 50^\circ$ (c, 1.0, water) (calculated for the anhydrous peptide from N content of solution). Air-dry crystals were analyzed. The data suggest the presence of both acetic acid and water of crystallization (Found C, 43.7, H, 8.1, N (Kjeldahl), 9.5%, amino N (Van Slyke, 4 min), 49% of total N. $C_8H_{16}O_4N_2 \cdot C_2H_4O_2 \cdot 2H_2O$ requires C, 44.3, H, 8.7, N, 9.4%, amino N, 50% of total N).

Ultramicrodetermination of the optical configuration of valine and leucine results with peptides from gramicidin

In preliminary trials it was found essential to purify the D-amino acid oxidase considerably before use, in order to eliminate impurities giving colours with ninhydrin. It was also found necessary to allow the enzyme reaction to proceed on paper that is thoroughly wet. The time of reaction should therefore be limited, since the amino acids in the spots diffuse freely under these conditions.

The following procedure was found satisfactory. The kidneys of freshly slaughtered sheep were worked up for D-amino acid oxidase according to Negelein & Brömel (1939).

level directly proportional to the amount of solution subjected to test. This indicates a yield of enzyme closely similar to that described by Negelein & Brömel.

For carrying out the assay, the peptides to be tested were hydrolyzed in excess of 6N HCl in sealed evacuated tubes for 48 hr at 110°. The hydrolysate was evaporated to dryness *in vacuo*, and the residue was dissolved in such a volume of water as would give a 0.2–0.1% (w/v) solution of the amino acid to be assayed. Spots of this solution (each 5 μ l) were placed on at least two different sheets of filter paper. Beside the spot on the first sheet were placed 5 μ l spots of solutions of different strengths of the amino acid undergoing assay. The spots were developed (after neutralization with λ H₂ vapour) as parallel one-dimensional chromatograms with *n*-butanol/water. On subsequent colouring with ninhydrin, the concentration of the test solution in respect of the amino acid being studied could be assessed. Solutions of the same amino acid were then made up, having the same total amino acid concentration, but different proportions of the D and L isomers. Solutions used were pure D, 25% L, DL, 75% L, and pure L. 5 μ l spots of each of these solutions were then placed beside the spot of test solution on the second sheet, and the chromatograms were developed with *n*-butanol/water after neutralization with λ H₂ as above. Care was taken to develop far enough to get wide separation of the leucine and valine spots. After development, the sheet of paper was dried in the air, and sprayed as evenly as possible with the enzyme solution (pH 8.3). It was important to wet the whole of the sheet during the spraying, otherwise the enzyme solution moved from one part of the paper to another, carrying the amino acid spots with it. The wet sheet was hung up at 37° in an atmosphere of O₂ rendered moist by bubbling through the 1/15 pyrophosphate buffer. After 2.5 hr the sheet was dried at 37°, and sprayed with 0.1% (w/v) ninhydrin in a mixture of 4 vol *n*-butanol and 1 vol acetic acid. The sheet was then hung in the hot room (37°) for the colour to develop (1–2 hr). Spots of pure D-leucine and D-valine were completely abolished by this treatment, whereas the L-isomers survived. It was possible to form rough estimates of the proportion of the isomers by comparing the test spot with the control spots. It was felt that estimates where L-isomers predominated were less accurate than those where D-isomers predominated. Results with the hydrolysates are shown in Table 4.

Table 4 Proportions of D- and L isomers of leucine and valine in hydrolysates of peptides from gramicidin

Fraction no	Peptide present	Leucine		Valine	
		% D	% L	% D	% L
26–30 (Synge, 1944)	Valylglycine	—	—	10	90
19–21 (peptides only)	Alanylvaline, leucylglycine	65	35	80	20
12	Alanylleucine	90	10	—	—
10	(?Alanylvalylleucine, ?Alanylleucylvaline)	90	10	60	40

The preparation was taken to 'Schritt 5'. The material precipitated at this step was not, however, dried, but was dialyzed free of $(NH_4)_2SO_4$ against several changes of Negelein & Brömel's pyrophosphate buffer (1/15, pH 8.3). The product from five kidneys, on diluting the bag contents with the same buffer solution to 50 ml and adding a saturating amount of coenzyme (flavin adenine dinucleotide) was used for spraying the paper chromatograms. 0.1 ml of this solution gave, in Negelein & Brömel's manometric test with DL-alanine, an uptake of 34 μ l O₂ in 10 min, the rate of uptake being at this

SUMMARY

1 Quantitative amino acid analysis of hydrolysates of gramicidin has shown that valine is liberated excessively slowly in acid hydrolysis. Previous valine figures are too low, with higher valine figures the data obtained with the fission products account satisfactorily for the elementary composition of gramicidin.

2 Further examination of the partial hydrolysate of gramicidin studied by Synge (1944) has led to isolation and recognition of the dipeptides D-leucylglycine, L-alanyl-D-valine and L-alanyl-D-leucine. The synthesis of the two last compounds is described.

3 Technical problems of fractionating the more complicated peptides in the partial hydrolysate are discussed, and some possible new approaches to the problem are suggested.

4 An ultramicro-method for assessing the optical configuration of individual amino acids has been developed and applied to leucine and valine. The amino-acids are separated on paper chromatograms, which are then sprayed with a solution of D-amino-acid oxidase from kidney.

5 The partial hydrolysis products of gramicidin so far recognized, and the optical form of the amino acids isolated from them and from gramicidin, are discussed with reference to Gregory & Craig's (1948) demonstration of the heterogeneity of gramicidin and to the problem of racemization, epimerization and inversion in the hydrolysis of peptides.

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A Study of the Acidic Peptides Formed on the Partial Acid Hydrolysis of Wool

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It has been accepted for many years that proteins consist of chains of amino acids in peptide linkage, and that the partial hydrolysis products of proteins consist of a complicated mixture of amino acids and peptides of various lengths. However, very few studies of the actual peptides present in the partial hydrolysis product of any given protein have, in fact, been made (see Synge, 1943). This has been due to the lack of methods for the separation and analysis

of complicated mixtures of peptides. Recently Consden, Gordon & Martin (1947) have developed such methods, and Consden, Gordon, Martin & Synge (1947) have applied these methods to the mixture formed by the partial acid hydrolysis of gramicidin S, many of the products in that mixture were identified.

The study reported here is of the vastly more complicated mixture produced by the acid hydrolysis of wool. The problem is confused in the case of wool by uncertainty as to the number of molecular species which are, in fact, present in the wool. Thus, wool has been broken up into fractions containing widely varying proportions of various amino acids (for

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bibliography and discussion see Goger, 1944, Lindley, 1947, Mercer & Rees, 1946), though Farrant, Rees & Mercer (1947) have obtained electronmicrographs for wool degraded by mechanical or enzyme action which suggests that both the proto fibrils and matrix material which together make up the cortical cells consist of similar approximately spherical particles. There is thus no certainty as to how many proteins exist in wool and it is clearly desirable that such products should be analyzed as soon as they can be separated. Since adequate methods of separation, or even of identification, are lacking at present, it has seemed best at this stage to examine whole wool to provide at least some experimental data which could be compared with those expected on the rather speculative hypotheses of the structure of wool which have already been put forward (Astbury, 1943).

The first objective of this investigation was to demonstrate in a preliminary way the presence of as many peptides as possible in partial hydrolysates of wool rather than to confine attention to the rigorous identification of a few products. The number of peptides involved is, in fact, so large that identification by classical means would be excessively laborious, even if it were possible.

The methods adopted have been preliminary separation by ion-exchange resins, followed by ionophoresis, which separates the peptides into groups having similar mobilities in an electrical field. These groups were examined by two dimensional chromatography, and numerous fractions were obtained. These fractions were hydrolyzed and the amino acids present identified. Further, those amino groups which are not involved in peptide linkage were destroyed by deamination and an examination was then made of the amino-acids remaining after hydrolysis. In many cases, the results obtained indicate that the fractions consist either of single peptides or of mixtures of a few simple peptides. These conclusions have been confirmed in some cases by comparison with synthetic peptides, which showed chromatographic and ionophoretic properties identical with those of the isolated material. In other cases where synthetic material was not available confirmation of assigned structures was obtained by comparison with nearly related peptides, which were found to have the expected differences in properties.

The only pretreatment which the wool had received was the removal of the tips which are known to contain cysteic acid (Consdon, Gordon & Martin, 1946b) and other decomposition products of cystine resulting from the action of light, air and weather (Race, Rowe, Speakman & Vickerstaff, 1938).

As previous work on partial hydrolysates of wool had given some indication of their extreme complexity (Gordon, Martin & Synge, 1941) the present

investigation was limited to acidic peptides containing dicarboxylic amino acids. A preliminary account of part of this work has already been given (Martin, 1946). The acidic rather than the neutral or basic fractions were investigated because acidic peptides can be more completely separated by ionophoresis: they show little or no tendency to be adsorbed by silica jelly and, since at neutrality they move against the direction of electroendosmotic flow, their net travel and therefore their separation in a jelly of given length is greater than that of either the neutral or basic peptides. Also, these peptides are easier to investigate by paper chromatography than those containing bases, which are liable to form elongated spots due to adsorption on the paper, or than the neutral peptides, some of the R_f values of which are known to be too high for convenient separation in the solvent systems at present commonly used.

The intensity of the ninhydrin spots of the amino-acids produced after hydrolysis of the peptides isolated from the paper chromatograms provides a rough means of estimating the amounts of the peptides. For this purpose the intensities were compared with those given by known amounts of amino-acids. As the ionophoretic band of glutamyl-glutamic acid contained no other peptide or amino-acid, it was possible to estimate this material directly, attempts to isolate the peptide from the hydrolysate, by chromatography on Amberlite IR-4 resin, were unsuccessful since it travelled with the aspartic acid. The other peptides moved, as expected, ahead of the glutamic acid since carboxyl groups in peptides are weaker than those in the corresponding amino acids (Edsall, 1943). In the case of glutamylglutamic acid this weakening is more than counterbalanced by the extra carboxyl group.

EXPERIMENTAL

Preparation of wool The wool used was New Zealand Corriedale 50's quality. After removing the tips of the fibres it was extracted with light petroleum (b.p. 40–60°) and rinsed in water.

Techniques and preliminary experiments Samples of wool hydrolyzed for 1, 3, 7 and 30 days were subjected to two dimensional chromatography. The 3 day hydrolysate was found to give the largest number of spots in the appropriate part of the chromatogram of an intensity suitable for analysis, and was chosen for further investigation. Two similar 3 day partial hydrolysates of wool were investigated, but only the second experiment is described here in detail. In the first experiment, ionophoretic fractions were investigated mainly by one dimensional chromatography and this served to identify a number of peptides. When the work was repeated using mainly two dimensional chromatography, many more peptides were identified owing to the much improved separation.

In the second experiment the successive stages in the analysis are shown in Fig. 1. A preliminary separation of the

acidic peptide fraction was carried out by chromatography on Amberlite IR-4 (Consden, Gordon & Martin, 1948). Most of the HCl was removed in the desalting apparatus (Consden, Gordon, Martin & Syngé, 1947) as in this way a much higher ratio of hydrolysate to resin could be used. After elution with acid from the Amberlite column, the solution was again freed from all but traces of HCl in the desalting apparatus. The material was then subjected to ionophoresis (Consden, Gordon & Martin, 1946a). As all the basic and neutral

with a wooden mallet. The trough, which was $100 \times 15 \times 0.48$ cm, was filled with the silicate solution with the lid already in position. For preparing buffered silicate at pH c 7, sodium silicate (93 ml) containing approximately 0.33 g SiO_2/ml was mixed with water (510 ml.) and *N* acetic acid added (201 ml). This solution set in 5 min. The jelly produced is fairly stiff and, after removal of the lid, gives a smooth surface from which satisfactory prints can be taken. Preliminary trials to obtain this setting time are necessary owing to variations in the silicate solutions. It was found that the setting time is considerably shortened by the presence of a large quantity of hydrolysate, hence the silicate concentration in the gutter was about 20% less than in the main jelly.

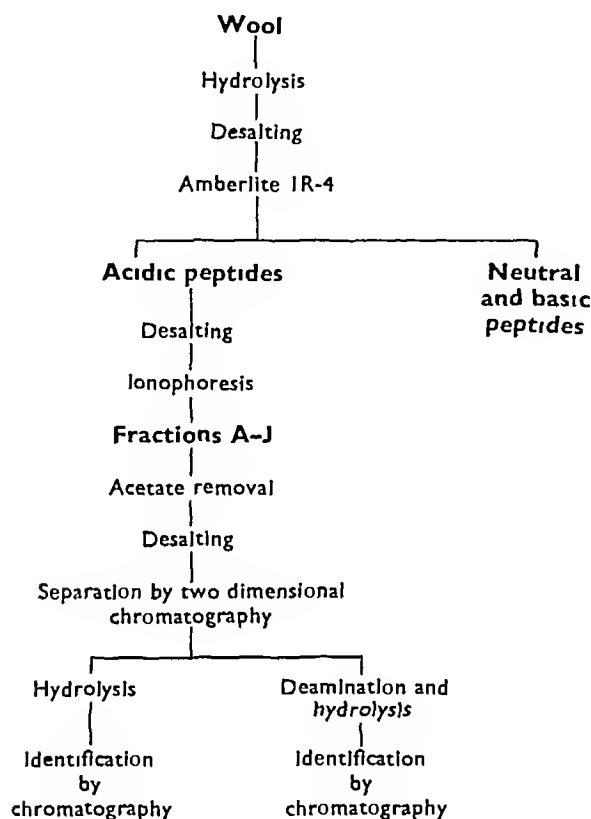


Fig 1 Successive stages of analysis of peptide mixtures

peptides and amino acids had been removed by the Amberlite treatment it was possible to carry out a successful ionophoretic separation of much more material than in the first experiment, where no preliminary fractionation had been employed. Finally, the ionophoretic fractions were worked up and examined by partition chromatography on paper, the solvents used being chosen after various preliminary trials in one dimension. In the following pages, 'phenol' denotes phenol saturated with water, used in an atmosphere of coal gas and NH_3 , the latter produced from a 0.3% aqueous solution on the floor of the chamber. 'Collidine' denotes collidine, saturated with water and used in an atmosphere containing a trace of diethylamine produced from a 0.1% aqueous solution. For ionophoresis, a modification of method B of Consden *et al* (1946a) was employed. Instead of using microscope slides to support the glass lid, the latter was made wide enough to rest on the sides of the trough as shown in Fig 2. In this way the jelly surface can be exposed at the end of the ionophoresis by sliding off the lid without removing either side bar. After a run of several days, it may be necessary to initiate the sliding by tapping

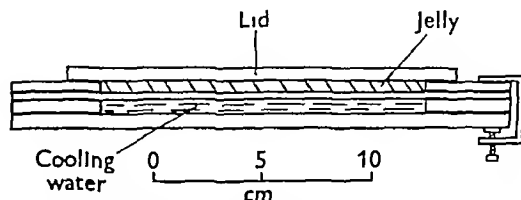


Fig 2 Cross section of ionophoresis trough showing position of lid

Investigation of 3-day hydrolysate. Wool (9 g) was hydrolyzed with 10*N*-HCl (100 ml) at 37° for 72 hr. HCl was removed from the solution by distillation *in vacuo* below 40° followed by treatment in the desalting apparatus. Much of the colour was thereby removed. After filtration the solution contained 1.29 g N (Kjeldahl). The solution was divided into two equal portions, I and II, each of which was treated as follows. After concentration *in vacuo* to 15 ml, the solution was adjusted to pH c 4 with *N* HCl (1.5 ml) and transferred quantitatively to a column of Amberlite IR-4 (height 30 cm, diam 1.5 cm) prepared and adjusted to pH 3-4 as described by Consden *et al* (1948). The column was then washed with water. The first 80 ml of eluate appeared to contain all the neutral and basic, but no acidic, amino acids and peptides, as shown by paper chromatography, a further 20 ml contained a negligible amount of N only. The column was then eluted with *N*-HCl, the removal of the pigment band serving as indication of complete elution. The N distribution of the two fractions are given in Table 1. To obtain blanks, the whole procedure was repeated omitting the hydrolysate. Blank (i) is the N washed out at pH 3-4 and blank (ii) is the N washed out by *N* HCl.

Table 1 *N* distribution of two portions of wool hydrolysate after Amberlite separation

	Portion I (N found)		Portion II (N found)	
	(mg)	(% of total)	(mg)	(% of total)
Neutral and basic fraction less blank (i)	448.6	70.4	440.6	70.0
Acidic fraction less blank (ii)	188.3	29.6	188.3	30.0
Total	636.9	—	628.9	—
Recovery (%)	(98.6)	—	(97.3)	—
Amberlite blank (i)	0.4	—	0.4	—
Amberlite blank (ii)	0.7	—	0.7	—

After removal of HCl in the desalting apparatus the solutions were concentrated *in vacuo*. Part (30%) of the acid fraction from I (Table 1) was next subjected to ionophoresis. The acidic peptide mixture was made up to 8 ml with silicate and acetic acid solutions and allowed to set in a gutter 1.1 cm wide cut out 3 cm from the cathode end of the jelly. After applying a potential of 270 V for 88 hr the lid was slid off and a paper 'print' taken and developed with

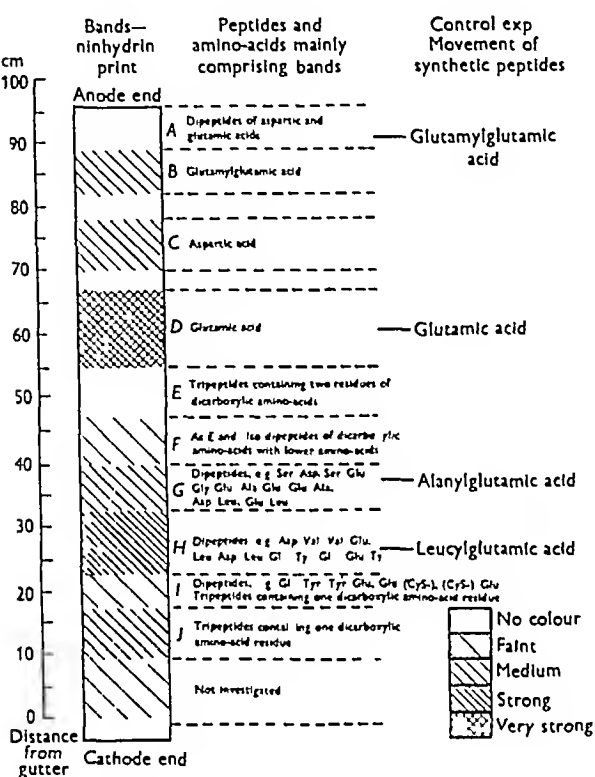


Fig 3 Ionophoresis of partial hydrolysate of wool

acidified ninhydrin solution (0.1% (w/v) ninhydrin in *n*-butanol containing 0.02 ml 10N HCl/ml). Nine bands, some of which were contiguous, of varying intensities were revealed and 10 fractions (A-J) were cut out as shown in Fig 3. The filtrates from each cut were freed from acetic acid and Na_2SO_4 as described by Consden, Gordon, Martin & Synge (1947). Next, the concentration of each fraction was gauged by one dimensional chromatography on small

samples, using phenol and collidine. Those fractions which separated insufficiently in phenol were tested in other solvents until a system giving adequate resolution was found.

Recoveries of amino acids from control mixture The mixture for this experiment consisted of glycine, DL serine, DL alanine, DL valine (3.2 mmol of each) and L glutamic acid (1.07 mmol), less of the latter being used because of its low solubility. This mixture was put through all the stages which had been used for the partial hydrolysate, omitting the Amberlite treatment. Recoveries after each stage were determined by estimation of N (Kjeldahl). The results are shown in Table 2.

Systematic examination of ionophoretic fractions Fractions A, B, C and D were investigated in the first ionophoresis only. A and B were found to be simple (R_F values 0.04 and 0.06, respectively, in phenol and 0.14 and 0.14, respectively, in collidine). These fractions were therefore analyzed by one dimensional chromatography using phenol. Fractions C and D were found to consist only of free aspartic and glutamic acids respectively.

Fractions E-J were investigated on duplicate two dimensional chromatograms on Whatman no. 4 paper as described by Consden *et al.* (1947) using 0.1% ninhydrin in *n*-butanol to reveal the full picture on one and 0.01% ninhydrin on the other to show the position of the spots to be further analyzed. Control one dimensional chromatograms of a complete hydrolysate of wool were developed parallel to that of the peptide mixture along two edges of each sheet. The control in the first solvent was cut off before development of the chromatogram in the second solvent. The chromatograms are represented in Figs 4-9. Collidine was the first solvent in all cases. The second solvent for fractions E, F and G was phenol. For fractions H and J, the second solvents were respectively *p*-cresol and *o*-cresol, each being used in an atmosphere of coal gas and NH_3 . The times of development in each direction are recorded in Figs 4-9. The amino acids, obtained by hydrolysis or by deamination followed by hydrolysis (separate chromatograms being used for each) of the extracted peptide spots, were identified by one dimensional chromatography. The solvents used for the latter were phenol or collidine. Where separations were inadequate in one solvent, the hydrolysates were divided and run in both. As a guide to the identification of the amino acids, control mixtures were chromatographed side by side with the treated peptide spots. For collidine, the control mixture consisted of lysine, arginine, serine, glycine, alanine, proline, valine, leucine, tyrosine and phenylalanine. For phenol, the control contained aspartic and glutamic acids,

Table 2 Recovery of amino acids from a control mixture of amino acids after various stages of analysis

Stage	N (mg) at start of stage	N (mg) at end of stage	Recovery (%)	Over all recovery (%)
Incubation	193.6	195.0	100.7	—
1st desalting	181.0	172.0	95.1	—
2nd desalting (after acidification with HCl)	168.3	162.0	96.3	—
Ionophoresis				
(a) Glutamic acid	4.65	4.22*	90.75	—
(b) Neutral compounds	55.75	48.73*	87.4	—
Acetate removal and desalting				
(a) Glutamic acid	3.27	3.11	95.1	79.0
(b) Neutral compounds	46.78	43.98	94.0	76.9

* Corrected for the N blank of the silica jelly

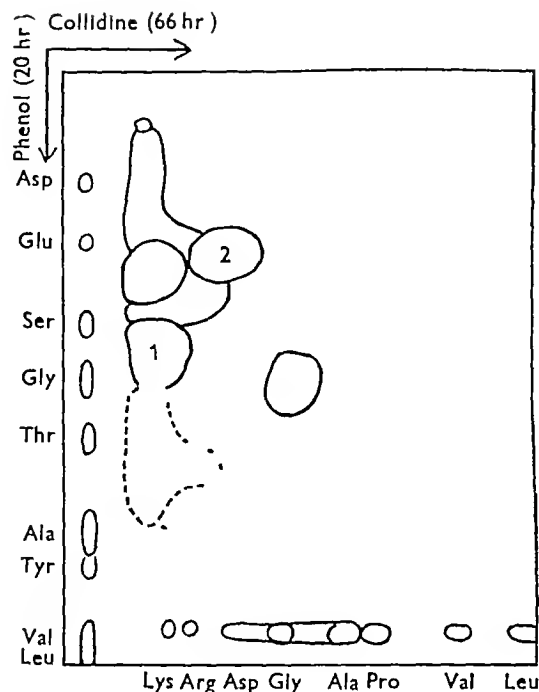


Fig 4 Two dimensional chromatogram of fraction *E* In Figs 4-9 the arrows show the direction of movement of the solvents (collidine being the first), the origin being at the small dotted circle. The spots of the ninhydrin treated chromatograms are shown in outline, the broken outlines represent the yellow 'salt' spot. The smaller spots along the edges of the sheets are those of the control mixtures, the positions of the amino acids of which are indicated by the abbreviations

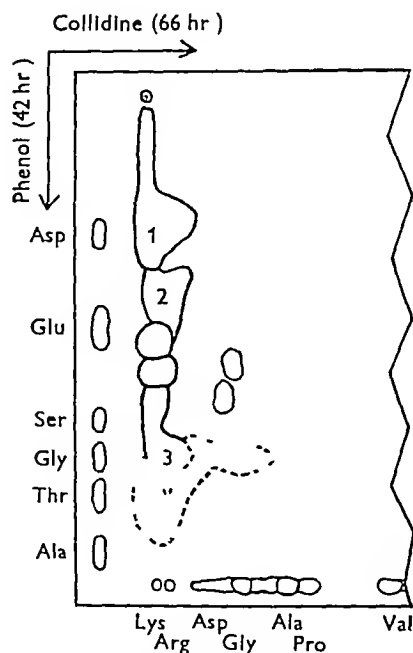


Fig 5 Two dimensional chromatogram of fraction *F*

serine, glycine, threonine, alanine, tyrosine, valine and leucine. Each solution was approximately 0.015M with respect to each amino acid and 3-5 μ l of each solution was used for each chromatogram. The results are given in Table 3

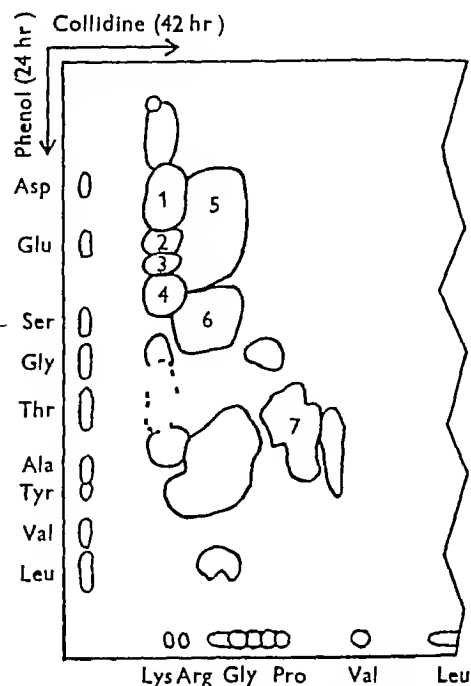


Fig 6 Two dimensional chromatogram of fraction *G*

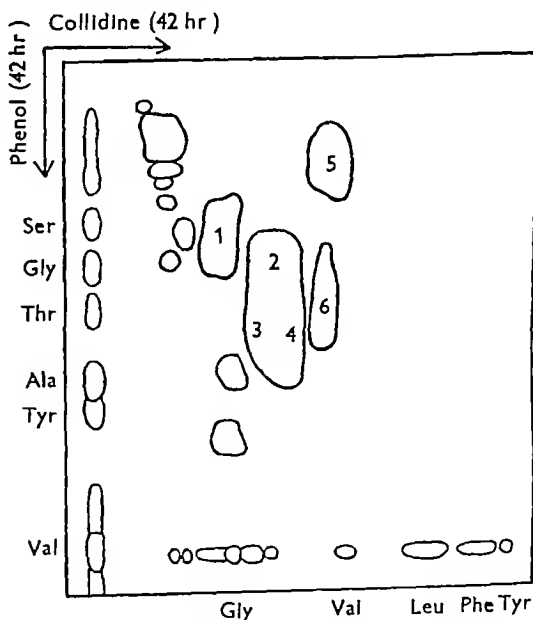


Fig 7 Two dimensional chromatogram of fraction *H*

As no attempt was made to distinguish between leucine, isoleucine and methionine, these amino acids are recorded as 'leucine'

The intensities of the ninhydrin spots are shown in Table 3 by x's which have the same significance as in the paper of Consden *et al* (1947), one x represents a rather faint to

moderate intensity and ? represents a very weak spot. Where a hydrolysate has been investigated in more than one solvent, the spot strength shown in Table 3 is an average

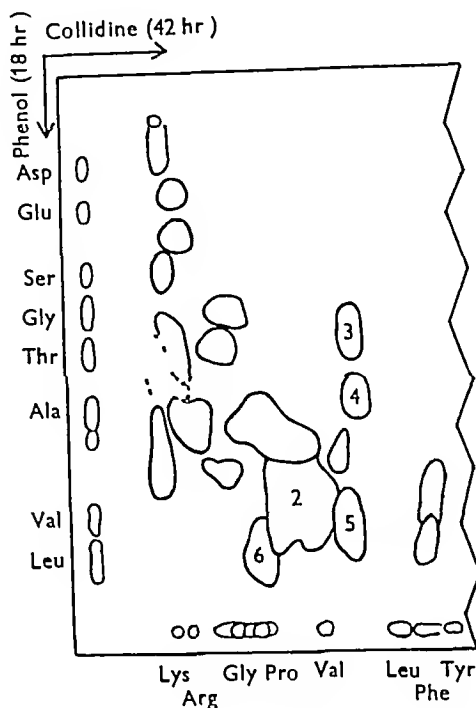


Fig 8 Two dimensional chromatogram of fraction I

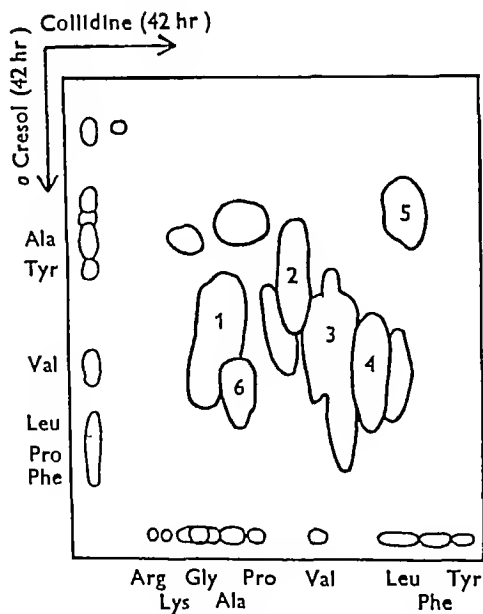


Fig 9 Two dimensional chromatogram of fraction J

The intensity of the ninhydrin spots was correlated with the amounts of amino acids present by developing control mixtures at various concentrations on one dimensional chromatograms in phenol and collidine (Table 4A). In order to estimate the losses sustained during the fractionation procedure, the control mixture used for the N recoveries (Table 2) was put through the complete procedure shown in Fig 1 omitting the Amberlite stage. The results are shown in

Table 4B. The intensities were found to agree well with the N figures given in Table 2.

After each fraction in Table 3 are shown those peptides which are considered to be present and those which may be present, but for which there is less definite evidence. Residues represented by a ? have not been included in the latter section unless they formed a considerable proportion of the whole fraction. A square bracket indicates that the order of the residues in the peptides has not been established. All the spots shown in Figs 4-9 were analyzed, but not all analyses are recorded here because in most of the cases concerned so many amino acids were obtained on hydrolysis that the results could not be interpreted. Spot 1 of fraction E (Fig 4) has been included as an example of the latter category. [The following abbreviations for amino acids or their residues are used in Table 3, Figs 4-9 and subsequently in the text, and are as proposed by Brand & Edsall (1947): alanine, Ala, arginine, Arg, aspartic acid, Asp, cysteine acid, (CysSO₃H), cystine (half molecule), (CysS), glutamic acid, Glu, glycine, Gly, leucine, Leu, lysine, Lys, phenylalanine, Phe, proline, Pro, serine, Ser, threonine, Thr, tyrosine, Tyr, valine, Val.]

Owing to repetition of the hydrolysis experiments, insufficient of fraction E was left for deamination. The deamination results for fraction F were obtained from one dimensional chromatograms developed in phenol for 66 hr, since the peptides were very slow in collidine (material of spot 1 after deamination and hydrolysis was lost). The occurrence of increased quantities of cysteine, aspartic and glutamic acids after deamination and hydrolysis of spot 3 is due to more material having been used for this stage. Spot 4 from a further chromatogram of fraction G was examined again. This spot was extracted and divided into two equal parts. One half was run as a mixed two dimensional chromatogram with the addition of 20 μ g L-glutamyl-DL-alanine, the other being used for a control chromatogram. Both were developed first in phenol (18 hr), followed by collidine (42 hr). On colouring with ninhydrin, both chromatograms were similar and showed two partially resolved spots having the R_F values of glutamylalanine and alanylglutamic acid (Table 6). In order to examine whether the serine residue is stable to deamination in dipeptides in which its amino group is protected, glycyl-DL-serine (60 μ g, Fischer & Roesner, 1910) was deaminated after extraction from a paper chromatogram which had been developed in phenol. Chromatography of the hydrolysate showed one spot only, which was serine.

The presence of tyrosylglutamic acid, leucylaspartic acid and leucylglutamic acid in fraction H was confirmed in the following manner: a mixed two dimensional chromatogram with 1.5% of fraction H and 10-20 μ g of each of these synthetic peptides were developed using the same solvents as above. As control equal amounts of the three synthetic peptides were applied to a second sheet which was developed at the same time in the same box. After colouring with ninhydrin the mixed chromatogram showed no significant difference from that obtained with fraction H alone. The chromatogram of the synthetic peptides showed only two spots, one corresponding in position to spot 5 (Fig 7) (tyrosylglutamic acid), the other to spots 2, 3 and 4 (Fig 7) (leucylaspartic acid and leucylglutamic acid).

Spots 2-5 of fraction I were not deaminated. The results for spot 1 were obtained from an elongated spot extracted from the top portion of a one dimensional chromatogram developed with phenol from the material of the first ionophoretic separation (p 549). Little cystine or cysteic acid was

Table 3 Analysis of fractions A-J

(Amino acids formed after hydrolysis (*H*) or deamination followed by hydrolysis (*D*) of peptide spots extracted from the chromatograms. Intensities of the amino acids formed are indicated by ×'s, — indicates not found. Square bracket indicate peptides of undetermined residue order.)

[illegible]

detected after hydrolysis of the corresponding spot on the two dimensional chromatogram, presumably owing to destruction of cystine during the chromatography. However, fraction *I* gave a very strong disulphide reaction with nitroprusside relative to the other fractions. The presence of cysteic acid after deamination and hydrolysis is considered to be due to oxidation of cystine by the oxides of nitrogen.

0.32N Na₂SO₄ for perfusing the electrodes. The bands shown on printing indicated a satisfactory separation of glutamyl glutamic acid. The aspartic and glutamic acid bands were satisfactory though still not widely separated, but the other peptide bands were decidedly less satisfactory than in the buffered jelly used in the main experiment. The fastest band observed on the paper print (glutamylglutamic acid) was

Table 4A Intensities of ninhydrin colours with various amounts of amino acids

	Amount of amino acid (μ mol)						
	0.24	0.12	0.06	0.03	0.015	0.008	0.004
Phenol							
Aspartic acid	x x x x x	x x x x	x x	x	?	—	—
Glutamic acid	x x x x x	x x x x	x x	x	x	?	—
Serine	x x x x x	x x x x	x x	x	x	?	—
Glycine	x x x x x	x x x x	x x	x	x	?	—
Threonine	x x x x	x x	x x	x	?	—	—
Alanine	x x x x x	x x x	x x	x	x	?	—
Tyrosine	x x x	x x	x	x	?	—	—
Valine	x x x x x	x x x x	x x	x	x	?	—
Leucine	x x x x x	x x x x	x x	x	x	—	—
Collidine							
Lysine	x x x x x	x x x	x x	x	?	—	—
Arginine	x x x	x x	x	?	?	—	—
Glycine	x x x x x	x x x x	x x	x	x	?	—
Serine	x x x x	x x	x	x x	?	?	—
Alanine	x x x x	x x x	x x	x	x	?	—
Proline	x x	x	x	?	—	—	—
Valine	x x x x x	x x x	x x	x x	x	?	—
Leucine	x x x x x	x x x	x x	x	?	?	—
Phenylalanine	x x x	x x	x	x	—	—	—
Tyrosine	x x x	x x	x	x	—	—	—

Table 4B Intensities of ninhydrin colours of amino acids on a paper chromatogram after passing a control mixture through complete procedure

(Amount of each amino acid at start was 0.4 μ mol)

Amino acid	Intensity of ninhydrin colour
Glutamic acid	x x x
Serine	x x x x
Glycine	x x x
Alanine	x x x
Valine	x x x x

Spot 3 of fraction *J* was not deaminated. In a mixed chromatogram of fraction *J* and leucylglutamic acid developed in collidine (42 hr), followed by *o*-cresol (42 hr), the leucylglutamic acid spot was separate from and vertically above spot 2, as expected from the R_F values.

Estimation of glutamylglutamic acid. A 3 day partial hydrolysate was prepared as above from another sample of the same wool, except that it was not desalted or fractionated with Amberlite IR-4. It contained 13.93 mg N/ml (Kjeldahl). The ionophoresis apparatus was filled with a solution made by bringing 4.3N-sodium silicate (52 ml) in water (350 ml.) to pH 7 with 0.9N H₂SO₄ (300 ml). This was allowed to set to a stiff jelly under a glass lid. The gutter contained 80 ml of a solution made by mixing 4.3N sodium silicate (0.45 ml), water (4.9 ml) and hydrolysate (3.6 ml.), the solution being adjusted to pH 7 with 0.9N-H₂SO₄ (0.05 ml). Thus, 44.8 mg N was contained in the gutter. Ionophoresis was carried out for 43 hr at 270 V using

3.2 cm wide, and only this width of jelly was cut out. The aspartic and glutamic acid bands (total width 8.5 cm) were cut out together. As a control, 8.5 cm of jelly was cut out well in front of the glutamylglutamic acid band. These fractions were filtered and desalted and each solution made up to volume. Table 5 gives the results of the N estimations. Chromatography of the glutamylglutamic acid band using phenol showed its homogeneity.

Table 5 Analysis of glutamylglutamic acid and dicarboxylic amino acids after ionophoresis

	N as % total N
Blank	0.05
Glutamylglutamic acid	0.6*
Free glutamic + aspartic acids	3.15

* As this cut was rather narrow, this figure must be considered as a minimum.

Attempted isolation of glutamylglutamic acid using Amberlite IR-4. The whole of the acidic peptide fraction (portion II, Table 1) was concentrated *in vacuo* and filtered. The solution was then transferred to a column of Amberlite IR-4 (length 30 cm, diam 1.5 cm) which had been equilibrated and washed with 0.003N HCl. Eluates were examined by partition chromatography using phenol. The results are shown in Fig 10.

R_F values of synthetic acidic peptides. These are given in Table 6. L-Glutamyl-L-glutamic acid was prepared according

to the method of Bergmann & Zervas (1932), L glutamyl glycine according to Grassman & Schneider (1934), glycyl L glutamic acid according to Fischer, Kropp & Stahlschmidt (1909), DL-alanyl L-glutamic acid according to Goldschmidt & Strauss (1929), L leucyl L glutamic acid according to Fischer (1907) and L leucyl L aspartic acid according to Fischer & Koenigs (1904). The preparation of the remaining peptides will be described in a later communication (Consden & Gordon, to be published).

(1 ml) The solutions were transferred to the holes and left to set. Two ionophoretic separations were carried out. In the first, glutamic acid (control) and leucylglutamic acid were used, in the second, glutamic acid (control), glutamyl glutamic acid and alanylglutamic acid were used. In both experiments, a potential of 200 V was applied for 17 hr, after which time the lid was slid off and paper 'prints' taken. The movement of each substance was measured from the ninhydrin print, which showed moderately strong purple

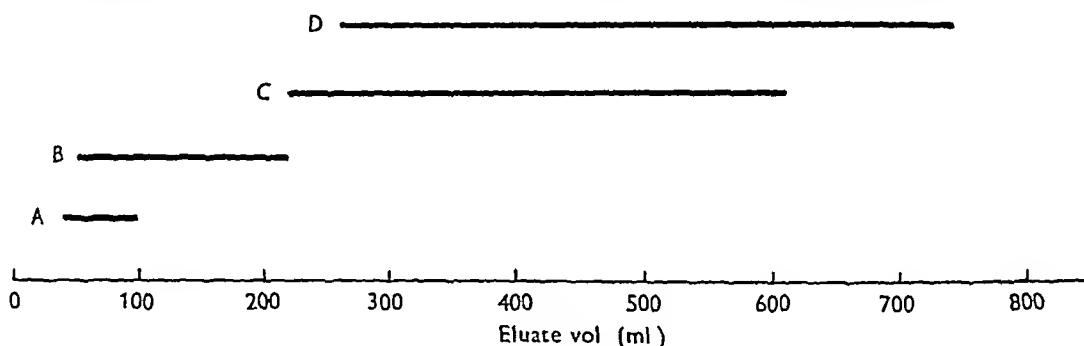


Fig 10 Chromatography of acidic fraction of partial hydrolysate on Amberlite IR-4 at pH 2.5. A, peptides of dicarboxylic amino acids with neutral amino acids, B, glutamic acid, C, aspartic acid, D, glutamylglutamic acid

Table 6 R_F values of synthetic acidic peptides in various solvents

Solvent	Phenol	Collidine	o Cresol	m Cresol	p Cresol
Addition	Coal gas NH_3 (0.3 %)	Diethylamine HCN	NH_3 (0.3 %)	NH_3 (0.3 %)	NH_3 (0.3 %)
L-Glutamyl L-glutamic acid	0.06	0.12	0.00	—	—
Glycyl L-glutamic acid (Y)	0.19	0.22	0.00	0.01	0.04
L-Glutamyl glycine	0.21	0.22	0.00	0.02	0.03
DL-Alanyl L-glutamic acid	0.37	0.24	0.00	0.02	0.06
L-Glutamyl DL-alanine	0.38	0.27	0.00	0.01	0.06
DL-Seryl L-glutamic acid (G)	0.19	0.25	0.00	0.00	0.00
		(streaky)			
L-Leucyl L-glutamic acid	0.66	0.39	0.06	0.12	0.31
L-Leucyl L-aspartic acid	0.55	0.39	0.04	0.10	0.23
L-Tyrosyl L-glutamic acid (GP)	0.35	0.50	0.02	0.04	0.07

Letters in brackets refer to colours given with ninhydrin. Purple. All the others gave purple colours.

Y = yellow, changing finally to purple, G = grey, GP = grey

Determination of ionophoretic rates of synthetic peptides of glutamic acid. The jelly for this experiment was prepared from acetic acid and sodium silicate as described above, but in this case its length was only 50 cm. Instead of a continuous gutter, a row of holes 0.6 cm diameter was made in the jelly

circles. The data are given in Table 7 and also plotted on Fig 3 after adjustment so that the glutamic acid bands superpose.

DISCUSSION

Interpretation of results

From the analysis of the spots, the presence of certain peptides can be inferred with certainty. However, we shall first discuss those results which are more difficult to interpret.

Fraction B (Fig 4). A possible hypothesis to explain the presence of amino-acids in very varying proportions in spot 1 is that alanine, serine, valine and leucine occur, each linked with two residues of a dicarboxylic amino acid as tripeptides. While such a hypothesis is not at variance with the expected chromatographic and ionophoretic behaviour of such substances, it cannot be proved without more refined separations than those so far achieved. Spot 2 is considered to be due to the tripeptides [Glu, Glu,

Table 7 Ionophoretic rates of some peptides of glutamic acid

Exp no	Substance	Diameter of spot (cm.)	Movement of centre of spot (cm.)
1	Glutamic acid	2.7	9.0
	Leucylglutamic acid	3.2	4.0
2	Glutamic acid	4.5	10.2
	Glutamylglutamic acid	2.6	15.4
	Alanylglutamic acid	4.0	6.4

near the cathode end. Each peptide (5–10 mg) was added to a partially neutralized sodium silicate solution (0.25 ml) until green to bromothymol blue (internal indicator). The partially neutralized sodium silicate was prepared by mixing 4.3N sodium silicate (1 ml), water (8 ml) and N acetic acid

Tyr] rather than dipeptides containing one glutamic acid residue (spot 5, fraction *H*, Fig 7) The ionophoretic rate of fraction *E* does not correspond to [Glu, Tyr] whose ionophoretic rate would presumably be similar to or slower than those of Glu Leu and Leu Glu which actually occur together with Glu Tyr and Tyr Glu in fraction *H* Moreover, the R_F values of spot 2, fraction *E* and spot 5, fraction *H* show a difference consistent with the presence of an extra glutamic acid residue In addition, no tyrosine-yielding spot occurs in the appropriate position in either of the chromatograms of the slower ionophoretic fractions *F* and *G* Since fraction *G* contains Glu Ala and Ala Glu (see below), it is unlikely that a dipeptide of glutamic acid with tyrosine (having a much larger molecular weight) will occur in fraction *E* Free glutamic acid may be present in spot 2 (which has the R_F values for this amino acid, Fig 4) owing to overlapping from fraction *D* In addition, peptides containing three or more residues of a dicarboxylic acid may well be present in this fraction

Fraction F (Fig 5) The presence of cysteic acid in the hydrolysate of spot 1 is probably due to oxidation of cystine peptides during the working up of fraction *F* Were peptides of cysteic acid with dicarboxylic amino acids originally present, their ionophoretic rates would presumably be similar to those of fraction *A* or *B* As Glu (CyS) and (CyS) Glu occurred in fraction *I*, it is considered that the faster moving fraction *F* contains tripeptides of cystine with two acidic residues (Asp and/or Glu), and not dipeptides Of the possible combinations in spot 2 [Glu, Gly, Glu] or [Glu, Ala, Glu] are regarded as being present, because the glutamic acid must be attached either to glycine or alanine, and Glu Ala and Ala Glu are excluded because these peptides have higher R_F values in phenol (fraction *G*, Table 6), and lower ionophoretic rates As glutamic acid decreases markedly, relative to glycine, after deamination it is probable that glutamic acid carries most of the free amino groups of the tripeptides

As the ionophoretic rates of the dipeptides of aspartic or glutamic acids with valine or leucine are much slower than that of this fraction, their presence is unlikely here but [Glu, Gly] or [Glu, Ala] may overlap from fraction *G* Hence tripeptides, Glu Glu with valine or leucine, are regarded as being present and similar tripeptides containing aspartic acid may be present

Fraction G (Fig 6) Spot 2 of fraction *G* is considered to contain Gly Glu because too much glycine, as judged by the intensity of the spots after hydrolysis, is present to be linked only with aspartic acid Similarly, spot 5 is considered to contain Ser Glu, but must also contain at least one of the dipeptides Ser Asp, Thr Asp It will be observed that the contiguous spots, 1 and 5, gave somewhat similar

results on analysis This was also true of the contiguous spots 4 and 6 The presence of the same substance in rather different areas of the chromatogram is presumably caused by the presence of interfering substances ('salt effect'), since material from spot 4, after removal of some of this interfering substance behaved as spot 6 material, and had R_F values in collidine and phenol similar to those for synthetic dipeptides alanylglutamic acid and glutamylalanine (Table 6) The 'salt effect' (of Consden & Gordon, 1948, Partridge & Westall, 1948) was observed in several of the ionophoretic fractions as a yellow zone (see Figs 4, 5, 6, 8) It must therefore be concluded that little reliance can be placed on R_F values unless the solution under investigation is free from salt or other interfering substances

Fraction H (Fig 7) Glu Leu is clearly present in cut 4 and hence is probably present in cuts 2 and 3 as these three cuts were all from one large spot (Fig 7) As phenylalanine gives a relatively weak colour with ninhydrin, (Table 4A), Phe Glu is recorded as being possibly present in cut 6, even though the phenylalanine spot obtained after hydrolysis was weak

Fraction I (Fig 8) The presence of proline in spot 2 is presumably due to an overlap of spot 6 (yellow) which was found to be free proline The presence of proline in fraction *I* can only be explained as the result of hydrolysis of a prolyl dicarboxylic acid peptide during working up at a stage subsequent to ionophoresis Spots 7 and 8 appeared to be respectively free aspartic and glutamic acids, which may be derived from the same peptide as the proline of spot 6 Spots 2, 4 and 5 are considered to consist mainly of tripeptides containing one residue each of a dicarboxylic amino-acid, since the ionophoretic rate of fraction *I* is slower than that of fraction *H* However, the possible presence of Glu Leu, Leu Glu, Glu Val and Val Glu cannot be excluded, owing to overlapping of the previous fraction Glu Ala and Ala Glu are excluded both on grounds of R_F values and ionophoretic rates (see Fraction *G*) The tripeptides [Glu, Ala, Ala] are excluded because they would be expected to have lower R_F values in phenol than spot 2 Glu Tyr and Tyr Glu cannot be present in spot 4 (which is equivalent to spot 5, fraction *H*) as this spot is well separated from spot 3, where they occur The section 'peptides possible' for this fraction in Table 3 has been omitted because of the large number which may be present

Fraction J (Fig 9) This fraction has a low ionophoretic rate compared with that of fraction *H*, the latter consists mainly of the dipeptides of aspartic or glutamic acids with valine or leucine The spots of fraction *J* also have high R_F values, which necessitated the use of *o*-cresol for the chromatograms (the R_F values of amino acids and peptides in

this solvent being lower than those in *m*- or *p* cresols, Consden, Gordon & Martin, 1944) Therefore this fraction is considered to consist mainly of tripeptides of aspartic or glutamic acids with two residues of higher neutral amino acids. Little additional information was obtained from the deamination of the spots, presumably because each consists of a mixture of tripeptides.

General conclusions

It can be seen (Fig 3) that ionophoresis separates acidic peptides, as expected, according to their charges and molecular weights, the peptides thus resemble the amino-acids (Consden *et al* 1946a). While it is well known that the depth of colour given by amino acids with ninhydrin on paper chromatograms is not an accurate measure of the amounts of these substances present (Polson, Mosley & Wyckoff, 1947), it is clear from the results presented here that the various peptides were present in widely differing amounts. Table 8 gives the relative proportions of those peptides considered to be certainly present, the data being derived from Table 3. These numbers

Table 8 *Dipeptides found with their approximate proportions*

(The figures represent the relative proportions of peptides listed in Table 3 as probably present. By adding the \times 's for each residue and multiplying by 100/(% of fraction used on each chromatogram), a number is obtained which is converted to N percentage by reference to Table 4A. Where peptides *A B* and *B A* (*A* and *B* being residues) are present, the relative proportion of the two has been estimated.)

Nitrogen (as mg/100 g total N of wool) present as peptides of the compounds in column 1 with aspartic or glutamic acids through the amino groups of the dicarboxylic acid

Amino acids present in wool (2 % or more of N)	(a) Free		(b) Combined	
	Aspartic acid	Glutamic acid	Aspartic acid	Glutamic acid
Aspartic acid	—	4	—	—
Glutamic acid	4	600	—	—
Serine	—	—	5	17
Glycine	—	5	—	8
Threonine	—	—	—	—
Alanine	—	29	—	27
Tyrosine	—	29	—	27
Valine	11	—	—	19
Leucine	11	21	19	51
Cystine	—	4	—	4
Proline	—	—	—	—
Phenylalanine	—	—	—	—

are only a very rough guide to the actual amounts present. Assuming the peptide losses to be similar to amino acid losses in the control experiments (Tables 2 and 4B), most of which occur during the last three stages shown in Fig 1, then the dipeptides identified, apart from glutamylglutamic acid, account for some 3 % of the acidic fraction. This

together with glutamylglutamic acid (2 %) and free glutamic and aspartic acids (11 %) account for a total of 16 % of the acidic fraction. Controls using synthetic peptides would be desirable, but these have not been done partly because of lack of material and partly because it is uncertain that the losses of the far simpler mixture, which one would have to use, would be at all comparable with the losses found with a hydrolysate. Nevertheless, it can probably be assumed that the peptide losses are higher than the amino-acid losses, owing to hydrolysis during the various stages, to formation of diketopiperazines and to other factors, in addition, since many of the chromatogram spots, which may well have contained dipeptides, could not be adequately identified, it is likely that dipeptides were originally present in far greater amounts than is indicated by the above figures.

Excluding the basic and dicarboxylic amino-acids, it has been found that of the nine amino-acids each known to be responsible for more than 2 % of the nitrogen of wool (Martin & Synge, 1941, Gordon, Martin & Synge, 1943a) five are linked to the α -carboxyl group and seven to the amino group of glutamic acid. Two were found to be linked to the α carboxyl and two to the amino group of aspartic acid (Table 8). However, as aspartic acid occurs only to the extent of approximately half the amount of glutamic acid, it is likely that many of its peptides were missed. Similar considerations apply to threonylglutamic acid, glutamylthreonine, glutamylphenylalanine and phenylalanylglutamic acid. Thus, the absence of these peptides from Table 8 does not necessarily imply their absence from the partial hydrolysates, only those peptides for which there is positive evidence being given in the table. Of the nine amino acids mentioned above, only proline has not been found linked to either of the dicarboxylic amino acids. This is probably due to the peptide linkage involving proline residues being particularly labile (fraction I). In this connexion it may be significant that Consden *et al* (1947) could not demonstrate the presence of prolylvaline in partial hydrolysates of gramicidin S, and that the synthetic peptide was found to be rather labile under the conditions of hydrolysis employed. A further reason for the failure to isolate a dipeptide containing a proline residue may be the ease with which such peptides change to diketopiperazines in aqueous solution (Fruton, 1948), these would be missed on the chromatograms, since they do not give colours with ninhydrin. The absence of glutamylserine, which contrasts with the presence of serylglutamic acid, is probably due to its special lability (cf Desnuelle & Casal, 1948) rather than to its absence from the peptide chain. A study of the kinetics of hydrolysis of these two peptides (cf Synge, 1945) would be specially desirable.

The best evidence that none of the peptides identified, and particularly valylglutamic acid, glutamylleucine, leucylglutamic acid, tyrosylglutamic acid, glutamylaspartic acid and glutamylglutamic acid, have arisen by recombination during the hydrolysis, is afforded by the observation by Consden, Gordon & Martin (unpublished work) that none of these dipeptides could be shown to be present in a partial hydrolysate of tyrocidine, which contains a proportion of dicarboxylic amino acids similar to that of wool (Gordon, Martin & Synge, 1943b), whereas aspartylglutamic acid and glutamyltyrosine were identified. Glutamylglutamic acid is the largest peptide component of the wool partial hydrolysate (about 10 % of glutamic acid nitrogen) and there is thus evidence that a substantial proportion of the glutamic acid residues are linked with other glutamic acid residues in the wool polypeptide chain.

Though the present results give no information as to long sequences of amino-acid residues, they appear to be of some value in relation to theories already put forward regarding the chemical structure of wool. Thus, Astbury (1943) has suggested, on the basis of chemical and X-ray data, that polar and non-polar

amino acid residues alternate along the peptide chains of wool. The identification of glutamylglutamic acid, aspartylglutamic acid, glutamylaspartic acid, tyrosylglutamic acid, glutamyltyrosine, serylglutamic acid and threonylglutamic acid, in amounts comparable to those of peptides of polar non-polar type, seem to make this hypothesis untenable (cf Martin, 1946).

SUMMARY

1 The acidic peptides from partial hydrolysates of wool, after separation by ionophoresis, have been analyzed by partition chromatography on paper.

2 Nineteen dipeptides of aspartic and glutamic acids have been identified in the hydrolysates. Evidence has been found for the presence of a number of tripeptides of undetermined residue order.

3 Glutamylglutamic acid was the peptide found in largest amount.

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Rancidity in Indian Butterfats (Ghee)

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It has long been realized that the analytical characteristics of deteriorated butterfats depart considerably from those of the fresh fats. It is the more surprising, therefore, that the changes in butterfat, or ghee, during ordinary rancidification—in loosely corked containers exposed to diffused daylight—have not been more intensively studied. Elsdon, Taylor & Smith (1931) studied commercial butters for very short periods and found increased Polenske values, while for butterfat Godbole & Sadgopal (1936) found decreased Polenske and increased Reichert values. Curl (1939) and Das Gupta (1939) excluded air from their samples during storage, while Narasimhamurty (1941) used commercial ghee samples whose purity was open to suspicion. In the present study, sets of samples of genuine butterfat from three different localities were chosen, and stored under the first-mentioned, more or less 'normal' conditions. The samples were not chosen at random, but represent characteristic products, one group from Indian buffaloes the butterfats of which were of roughly normal Reichert value (high for a buffalo) and normal iodine value, another from buffaloes heavily fed with cottonseed, a practice common in many dry areas in India, and a third from cows in pasture, such as are produced in any well-kept dairy. It is possible, therefore, to draw comparisons between one set and another and also between individuals in the same group, a feature which will be appreciated during the discussion.

A study of the changes in the characteristics alone, while it enables certain conclusions to be drawn, would it was felt be somewhat restricted and it was decided therefore to isolate and study the free fatty acids produced by rancidity in the three batches of ghee. The products of spoilage of fats, as hitherto enumerated and sometimes isolated, include *inter alia* ketones, aldehydes, peroxides, hydroperoxides, ozonides, hydroxyacids and fatty acids. Whilst clearly the terms rancidity and acidity due to free fatty acids are not equivalent, it will be conceded that free fatty acids, especially over long periods as in the present instances, certainly form the largest proportion of the rancidity products, and may amount to as much as 20% of the rancid fat, while peroxides (calculated as oleic peroxide) may approach only 2% and aldehydes and ketones, according to Munding (1930), 'can be detected'

The formation and composition of these fatty acids therefore represents a major factor in fat spoilage, especially in rendered fat where lipolytic complications may tend to be of subsidiary occurrence.

EXPERIMENTAL AND RESULTS

Methods

The three sets of Indian ghee chosen for study were (1) eleven samples from buffaloes of the Agricultural College, Kirkc, initially of high Reichert and low iodine values, a characteristic sample of which had been analyzed earlier for fatty acid and glyceride composition by Achaya & Banerjee (1940), (2) ten samples from Porbandar buffaloes (heavily fed with cottonseed under arid conditions) of extremely low Reichert and high iodine value, a representative sample had been studied earlier, (3) twelve samples from cows of the Cattle Farm, Hosur, as representing normal, highgrade cow ghee.

All the products were in excellent condition when originally analyzed, but, when the present study was undertaken, had been maintained in storage under the conditions mentioned earlier for 3–4 years. The characteristics were determined in the usual way: acidities are expressed as acid values (mg KOH/g fat), peroxide values as ml 0.002N $\text{Na}_2\text{S}_2\text{O}_8$ /g fat, and refractive indices in terms of butyrefractometer degrees at 40°.

After the characteristics had been determined, the individual samples in each lot were mixed and the free fatty acids extracted successively with boiling ethanol by thoroughly mixing, cooling till the fat solidified and drawing off the supernatant ethanol. Owing to the low concentration of total fatty acids present in the ethanol (maximum 7%) and to mutual solubility effects between the fatty acids there is little reason to fear that stearic or higher acids may thus have been lost by deposition with the neutral fat in the solid phase during the ethanol extractions. After five or six extractions (when typical acidities of the orders, 23, 11, 5.7, 4.2, 7.1, 8% were left in the separated fat phases), the ethanol extracts were mixed, exactly neutralized with conc. KOH, the ethanol driven off and the soaps thoroughly extracted with ether to remove any adventitious neutral fat. Tables 1 and 2 give a summary of the results obtained.

Analytical results

The changes in the analytical figures are in general extremely well defined, and very similar in the three groups. Of the thirty-three samples, only three show a fall (and these extremely small) in Reichert value. The increases in Polenske value are more striking, amounting sometimes to four times that of the fresh

fats but averaging about double. With a single exception the iodine values, as to be expected, showed diminution to varying extents which are roughly parallel with the production of free acidity in the individual specimens concerned. Large increases in the saponification values are almost always apparent, while the refractive indices vary erratically.

The acidities and peroxide values demand separate consideration. It is abundantly clear that, in advanced rancidity, the peroxide values are little indication of the extent of its progress as perceived organoleptically. A curious feature associated with these samples may be mentioned. Some of the Kirkee samples were coloured quite bright yellow, while the others were almost colourless, and it is in the former samples that the peroxide and acid values are very small. This would lead one to speculate on the possible anti-oxidative nature of the carotene in the specimens—further supported by the generally low acid and peroxide values of the bright yellow cow ghees from Hosur, and the contrasting high values of the extremely white Porbandar buffalo ghees.

value (usually also associated with large increases in peroxide value) are almost invariably accompanied by similarly large increases in Reichert, in Polenske and in saponification values and, most significantly, by correspondingly large falls in iodine value. The peroxide values are very small in actual amount (1–2 % of the total fat), and, since they are generally recognized in rancidity as precursors of further breakdown products, it is perhaps natural to expect them in quantities proportional to the fatty acids already present, fresh peroxide being formed as part of it was converted to further products. Increases in acidity run concurrently with losses in iodine value in butterfat, the latter is a measure mainly of oleic glycerides, and it follows that oleic glycerides are therefore converted largely into free acids. Further evidence that this is the case is obtained by plotting the loss in iodine value of each sample against the acidity (calculated as a percentage of the mean molecular weight of the free acids (saponification equivalents) as shown in Table 2). The resulting smooth curves (Fig. 1) appear to be characteristic of an autocatalytic reaction, the agent being perhaps the peroxides initially formed or even the free acids

Table 1 *Changes in the analytical characteristics of ghees during development of rancidity*

Characteristic	Original samples		Rancid samples		Change due to rancidity	
	Range	Average	Range	Average	Range	Average
Kirkee, buffalo ghees, 11 samples, normal pasture feed						
Acid value	Negligible	Negligible	5.3–52.3	24.9	+5.3–+52.3	+24.9
Saponification value	223.9–239.3	228.9	227.5–261.0	243.6	–2.2–+33.5	+14.7
Iodine value	25.7–31.1	28.9	6.9–28.3	18.1	–24.2–+0.8	–10.8
Butyrefractometer reading (40°)	39.2–43.1	41.9	40.3–42.6	41.7	–1.6–+1.3	–0.2
Reichert value	25.7–39.1	32.1	32.2–40.7	36.4	–0.6–+12.3	+4.3
Polenske value	1.1–2.7	1.7	1.7–4.4	2.9	–0.3–+2.9	+1.2
Peroxide value	Negligible	Negligible	0.7–71.6	34.7	+0.7–+71.6	+34.7
Porbandar, buffalo ghees, 10 samples, high cottonseed feed						
Acid value	Negligible	Negligible	11.6–24.1	18.2	+11.6–+24.1	+18.2
Saponification value	200.8–224.0	213.3	213.0–250.2	226.9	–2.6–+29.5	+13.6
Iodine value	30.6–39.9	35.4	14.0–28.3	19.0	–22.1–+7.4	–16.4
Butyrefractometer reading (40°)	43.8–46.9	45.6	43.8–46.4	45.1	–1.4–+0.9	–0.5
Reichert value	14.5–33.6	21.0	18.9–37.0	24.9	–0.7–+5.8	+3.9
Polenske value	0.4–2.4	1.1	1.1–3.7	1.9	–1.0–+2.8	+0.8
Peroxide value	Negligible	Negligible	19.4–103.4	54.0	+19.4–+103.4	+54.0
Hosur, cow ghees, 12 samples, normal feed						
Acid value	Negligible	Negligible	5.3–14.8	10.1	+5.3–+14.8	+10.1
Saponification value	213.2–226.3	221.4	227.6–238.3	232.5	+4.5–+22.6	+11.1
Iodine value	34.5–39.2	37.1	22.2–35.0	28.8	–15.2–+1.8	–8.3
Butyrefractometer reading (40°)	43.8–45.0	44.3	43.7–45.2	44.3	–1.1–+0.7	Nil
Reichert value	23.8–26.2	24.8	27.9–30.2	28.9	+3.1–+5.2	+4.1
Polenske value	1.2–2.1	1.6	2.0–3.0	2.5	+0.4–+1.6	+0.9
Peroxide value	Negligible	Negligible	15.0–48.5	32.5	+15.0–+48.5	+32.5

The most outstanding feature of Table 1, however, is the marked parallelism between the alterations in each characteristic, suggesting that they all proceed from a single general cause. Large increases in acid

themselves. Davies (1941) studied somewhat empirically the free fatty acids of rancid butter and found them to be mainly composed of oleic and lower acids, while Rangappa & Banerjee (1946) found

broadly similar features in the acid fractions extracted from market samples of butterfat it is often the case, however, that these are made from butter that

values of the de acidified fats studied in the present work were in all three cases extremely close to the original values for these fats when fresh, indicating that these glycerides had largely remained intact. All these features appear to point to one fact—a mainly

Table 2 Free fatty acids from united samples of rancid ghees

	Mixed rancid ghee	Mixed neutralized ghee	Free fatty acids isolated from rancid ghee
	Kirkee, buffalo ghee		
Appearance	Soft, heavy odour	Hard, bland odour	Soft, yellow, crystalline solid
Melting point (°)	34.3	40.3	32.6
Solidification point (°)	27.8	32.8	31.6
Butyrefractometer reading (40°)	41.7	42.6	29.4
Acid value	23.0	1.8	—
Saponification value	243.6	221.4	320.3
Saponification equivalent	230.2	253.4	175.2
Iodine value	19.1	15.9	26.4
Reichert value	35.9	26.9	119.8
Polenske value	3.1	1.7	13.5
Peroxide value	30.7	16.9	—

Porbandar, buffalo ghee

	Pasty, acid odour	Hard and granular, bland odour	Just solid, amber coloured, crystalline
Appearance			
Melting point (°)	38.7	39.2	32.7
Solidification point (°)	34.5	36.0	29.6
Butyrefractometer reading (40°)	44.4	45.2	30.6
Acid value	23.1	4.6	—
Saponification value	237.1	230.3	294.4
Saponification equivalent	236.6	243.5	190.6
Iodine value	20.6	20.3	22.5
Reichert value	28.8	24.9	49.3
Polenske value	2.7	1.3	13.2
Peroxide value	28.5	17.7	—

Hosur, cow ghee

	Pasty, rancid but not acid odour	Soft, faintly granular, bland odour	Very soft, dark amber in colour, crystalline
Appearance			
Melting point (°)	35.8	34.2	28.0
Solidification point (°)	36.4	36.6	28.0
Butyrefractometer reading (40°)	43.7	44.4	37.1
Acid value	10.6	3.1	—
Saponification value	232.1	228.8	293.6
Saponification equivalent	241.6	245.3	191.1
Iodine value	29.6	30.0	29.1
Reichert value	28.8	26.5	67.6
Polenske value	2.5	2.1	13.0
Peroxide value	32.7	26.8	—

has deteriorated considerably so that the characteristic rancidity products of the latter are liable to be carried over. Again, the Reichert and Polenske

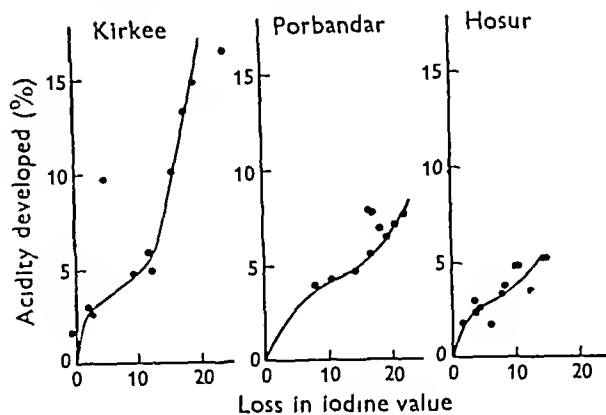


Fig. 1 Rise of acidity accompanying loss in iodine value during rancidity

oxidative mechanism producing free acidity in butterfat during spoilage, as opposed to a lipolytic one (possibly somewhat selective towards oleic and lower fatty glycerides) in butter

Detailed study of the free fatty acids

The free fatty acids (thus isolated in the form of dry soaps) were now analyzed for component fatty acids, the Kirkee soaps were divided into two lots and analyzed using the lead-salt separation technique and the low temperature crystallization procedure (using 10 ml of ether/g of acids at -45°), respectively, for the partition of the acids non-volatile in steam (Hilditch, 1947a) the results being calculated on the basis of normal straight-chain fatty acidity. The analyses differed substantially and whilst this may partly be due to the small amounts available for analysis, a more important cause appeared from subsequent work to lie in the presence of short chain dicarboxylic acids and *n*-octane-1-carboxylic acid (produced as scission products of oxidation from oleoglycerides, the presence of which was not at first realized). The dicarboxylic acids appeared in both the soluble and deposited portions during the lead salt separation, whereas the crystallization procedure gave a more straightforward separation and was consequently employed for the other two analyses. Details and results obtained by the alternative lead-salt method of separation will not therefore be recorded here.

When the presence of significant proportions of *n*-octane-1-carboxylic acid and also, apparently, dicarboxylic acids was appreciated, an attempt was made to identify these and also, by modifying the ester fractionation procedure, to obtain an approximate measure of the amounts present. For this

purpose the following methyl ester fractions were mixed and carefully refractionated through the electrically heated and packed column from Porbandar rancid buffalo ghee—ester fractions from the steam-volatile acid group (saponification equiv 134.9–182.3) and from the acids soluble in ether at -45° (saponification equiv 128.1–164.3), from Hosur rancid cow ghee—ester fractions from the steam volatile acid group (saponification equiv 131.2–198.8) and from the acids soluble in ether at -45° (saponification equiv 129.7–178.1). The boiling points (at about 0.2 mm pressure) rose steadily from 54 to 124° , but the equivalents of the fractions obtained were, in order of increasing boiling point 148.3, 153.6, 164.2, 169.4, 133.4, 119.8, 118.4, 131.8, 170.8, 177.0, 201.0 and 243.3. It was evident that esters of dibasic acids were present (e.g. dimethyl azelate has boiling point 140 – $141^{\circ}/9$ mm). These were therefore removed by washing the remixed free acids with light petroleum, filtering, and isolating any dissolved dibasic acids by cooling the monobasic acids several times in light petroleum or ether solutions at temperatures down to -20° . Finally there were obtained 12.9 g of crude dibasic acids and 12.8 g of monobasic acids. The latter were re-esterified and fractionally distilled, and now showed no discontinuity in the sequence of saponification equivalents which rose steadily as follows 162.1, 169.1, 169.2, 180.8, 188.2, 198.1, 212.6, 230.1 and 284.4. The determined figures for the acids present in this group of esters fractionated after removal of the dibasic acids, were *n* heptane 1 carboxylic, 7.6, *n* octane 1 carboxylic, 18.1, *n* nonane 1 carboxylic, 22.5, lauric, 29.5, myristic, 8.8, palmitic, 6.2 and stearic, 7.3% (w/w), whilst typical estimates of the total esters (including dicarboxylic esters calculated as dimethyl azelate) present in two of the mixed esters were as follows *n* pentane 1 carboxylic, 2.3, 0.8, *n* heptane 1 carboxylic, 14.0, 7.5, *n* octane 1 carboxylic, 18.5, 19.9, azelaic, 29.8, 31.7, *n* nonane 1 carboxylic, 22.8, 22.9, lauric, 7.5, 8.3, myristic, 4.8, 8.9, and palmitic, 0.3% (w/w).

By crystallizing the crude dibasic acids (of above) repeatedly from CHCl_3 at room temperature they were ultimately separated into two fractions, (i) about 80% of the crude acids, which now melted at 86 – 96° and had an equivalent of 95.4 (azelaid acid 94.0), and (ii) about 20% of acids which melted at 106 – 108° and had an equivalent of 81.8. It therefore appeared that the greater part of the dicarboxylic acids consisted of azelaic acid, $\text{COOH}(\text{CH}_2)_7\text{COOH}$, but it is possible that small proportions of dibasic acids of higher (?sebacic) and lower mol wt than azelaic acid were also present amongst the products of rancidity.

The presence of *n*-octane 1 carboxylic acid was confirmed by its identification as the *p* toluidide, this derivative being selected because of the convenient discriminate melting points in this range of the homologous series. For comparison, the *p* toluidides of authentic specimens of butyric, *n*-pentane, *n* hexane, *n*-heptane, *n*-octane and *n* nonane 1 carboxylic acids were also prepared. The sources of these acids were as follows: butyric and *n* hexane 1 carboxylic acids were purchased from commercial sources (equiv 88.8 and 128.8, respectively). *n* Pentane and *n* octane 1 carboxylic acids were prepared by oxidizing a concentrate of methyl oleate and linoleate (iodine value 103.1) with KMnO_4 in acetone, isolating by distillation in steam the monobasic acids formed, and distilling the latter through a fractionating column when *n* pentane and *n* octane 1 carboxylic acid fractions were obtained in a state of purity (equiv 116.0 and 157.6, respectively). *n*-Heptane and *n* nonane 1 carboxylic acids were obtained from the respective methyl esters iso-

lated by fractional distillation of the mixed methyl esters of coconut oil acids (equiv 145.8 and 168.5, respectively).

Table 3 *p*-Toluidides of lower normal fatty acids

Acid used	Equivalent of acid		Melting point of <i>p</i> toluidide ($^{\circ}$)	
	Found	Calc	Found (present work)	Robertson (1908) (1919)
<i>n</i> Butyric	88.8	88.0	74.5–75.5	74 75
<i>n</i> Pentane-1-carboxylic	116.0	116.0	71.5–72.5 (rising)	75 73
<i>n</i> Hexane 1-carboxylic	128.8	130.0	78.5–79.5	80 80
<i>n</i> Heptane 1-carboxylic	145.8	144.0	68.0–68.5	67 70
<i>n</i> Octane 1-carboxylic	157.6	158.0	83.0	81 84
<i>n</i> Nonane 1-carboxylic	168.5	172.0	76.5–78.0	80 78

The fatty acids (about 1.0 g) were refluxed under water condenser with 100% excess of SOCl_2 for 1 hr, and excess reagent removed by gentle suction for 15 min. 100 ml of dry ether were now added to the mixture, 2.5 mol of *p* toluidine introduced and washed in with a little ether. A bulky precipitate of *p* toluidine hydrochloride immediately formed, the whole was vigorously refluxed for 2 hr, more ether being added if necessary. The contents of the flask were washed with water and dilute HCl in a separating funnel, then with dilute KOH and finally with water. The extracts were dried, filtered, the product refluxed in hot ethanol with animal charcoal and crystallized from pure methanol or ethanol. Table 3 gives a summary of the properties of the *p* toluidides prepared from the authentic acids. The products were highly crystalline compounds, but even after repeated crystallization still melted over a range of about 1 – 2° , this, however, is not a drawback for the present purpose since the melting points of successive members are well apart. The melting points compare well with those recorded earlier in the literature (Robertson, 1908, 1919) except for the *n* pentane 1 carboxylic derivative which was available in quantities too small for further crystallization, and was probably still impure. The present starting materials were in general probably of greater purity than those available to Robertson (1908, 1919).

From the ester fractions obtained after the removal of dibasic from monobasic acids (of col 1) two fractions were chosen for preparation of derivatives, their respective saponification equivalents being 169.2 and 188.9 (cf methyl *n* octane 1-carboxylate, 172, methyl *n* nonane 1 carboxylate, 186). The derivatives were obtained in theoretical yield, and after three crystallizations from ethanol, each gave highly crystalline products melting at 83.0 and 74.5° , respectively, undepressed on mixing with the corresponding pure toluidides and pointing unmistakably to the presence of *n* octane and *n* nonane 1 carboxylic acids in the original fatty acids.

Another point of interest was to study whether fatty acids of lower molecular weight than butyric acid were produced during the course of oxidative rancidity. This was investigated by two methods: first, by isolating the acids from the titrated soaps in the ether-extracted aqueous solution from

the steam distillate. These soaps were evaporated to dryness on a water bath and then on a sand bath at 150°, and finally kept in a desiccator until required for use. They were then distilled with dry H_3PO_4 (preheated to 150° for 3 hr), and the distillate stood over anhydrous Na_2SO_4 for several days. About 0.8 g of these acids was now fractionally distilled in a microfractionation apparatus and yielded fractions as follows:

Wt (g)	0.0883	0.0812	0.0838	0.0738
Equiv	68.3	64.2	85.0	83.1
Wt (g)	0.0379	0.2200	0.1803	
Equiv	84.0	105.3	97.3	

The first two fractions clearly contained acetic acid (equiv 60), the remainder had acid equivalents corresponding with butyric (88.0) or mixtures of this with *n*-pentane-1-carboxylic (116.0) acid, the proportions of acetic butyric are of the order 1:3.

The second method used was a chromatographic separation by the procedures of Martin & Synge (1941), Elsdon (1946), and Rawsey & Patterson (1946), employing moist silica gel as adsorbent and $CHCl_3$ containing *n*-butanol as eluent. Two bands were obtained with ease using a 1% butanol $CHCl_3$ eluent, and when this was followed by 5% butanol $CHCl_3$, a third band also appeared, but tended to become diffuse as it moved down the column. The silica gel was therefore removed in zones, placed in water, and, after aeration to remove $CHCl_3$, was gently steam distilled, the distillates titrated and calculated to acetic, butyric and *n*-pentane-1-carboxylic acids respectively, when the relative proportions by weight were found to be 2:6:3. This accords extremely well with the proportions 1:3 for acetic butyric obtained by the former method.

It should be pointed out, however, that these results do

not demonstrate unequivocally that acetic acid is one of the products of a process of oxidative rancidity, since the quantities observed were so small that the possibility that the acetic acid arises adventitiously (e.g. from traces of acetic esters still left in the ethanol used in the course of the work in spite of drastic purification) must not be overlooked.

In the course of the above work, therefore, the following have been established: (i) The discrepancy between the lead salt method of analysis and the low-temperature crystallization technique leading to the preferred use of the latter subsequently. (ii) The proof of the presence of dibasic acids, mainly azelaic, but with traces of both higher and lower homologues. (iii) The clear characterization of *n*-octane- and *n*-nonane-1-carboxylic acids by the preparation of their *p*-toluidide derivatives, with the incidental preparation of reference *p*-toluidides of several saturated fatty acids. (iv) The presence of acetic acid (though perhaps of adventitious origin) in the free fatty acids, as demonstrated by fractional distillation and by chromatographic separation.

DISCUSSION

Table 4 shows the results of the analyses of the free fatty acids of the rancid ghees. In the analyses of the Porbandar and Hosur samples it has been possible to make an approximate allowance for the *n*-octane-1-carboxylic and dibasic (as azelaic) acids which were shown to be present. Unfortunately shortage of material prevented this correction in the case of the Kirkee fatty acids.

Table 4. Composition of free fatty acids from rancid ghees

	Percentage (w/w)			Percentage (molar)		
	Kirkee (U)	Porbandar (C)	Hosur (C)	Kirkee (U)	Porbandar (C)	Hosur (C)
Saturated						
Acetic	?	0.6	?	?	1.9	?
Butyric	10.9	3.5	4.0	21.5	7.5	8.7
Caproic	13.9	4.6	2.8	20.8	7.5	4.6
Caprylic	9.7	4.3	5.2	11.6	5.7	6.9
<i>n</i> -Octane-1-carboxylic	?	6.1	7.4	?	7.4	9.0
Capric	4.7	9.0	9.8	4.8	9.9	10.9
Lauric	4.3	3.0	4.0	3.7	2.9	3.8
Myristic	4.5	6.8	4.1	3.4	5.7	3.5
Palmitic	20.0	15.4	9.9	13.5	11.4	7.4
Stearic	3.1	4.3	1.3	1.9	2.9	0.9
Arachidic	—	—	0.8	—	—	0.5
Azelaic	?	9.4	11.4	?	9.5	11.5
Unsaturated						
Nonene-1-carboxylic	0.8	0.9	0.7	0.8	1.1	0.8
Undecene-1-carboxylic	0.4	1.3	0.3	0.3	1.2	0.3
Tridecene-1-carboxylic	1.8	0.6	1.0	1.4	0.5	0.8
Pentadecene-1-carboxylic	6.7	9.1	10.2	4.5	6.8	7.7
Oleic	3.6	3.3	6.1	2.2	2.2	4.1
Unsaturated residues	15.6	17.8	21.0	9.6	15.9	18.6
Unsaturated residues		Kirkee	Porbandar	Hosur		
Equivalent		224.7	213.2	215.2		
Iodine value		68.3	53.9	59.3		

U = Uncorrected for acetic, *n*-octane-1-carboxylic and azelaic acids. C = Corrected for acetic (Porbandar only), *n*-octane-1-carboxylic and azelaic acids (Kirkee and Porbandar).

The comparatively small amounts of experimental material available, together with the complexity of the fatty acid mixtures and the complication introduced by the presence of some dicarboxylic acid, causes the figures in Table 4 to be only broadly indicative in character. Nevertheless, there is considerable general resemblance between the proportions of the various component acids, which are apparently largely independent of the more widely variable nature of many of the acids in the three corresponding original ghees (cf Achaya & Banerjee, 1946). Thus, the Porbandar samples were buffalo ghees of very low Reichert (14–20) and high iodine (30–40) value, high stearic and low palmitic acid content, in each of these respects differing from the Kirkee samples. The data in Table 4 thus suggest that the products of free acid and oxidative rancidity from all three ghees are much the same in type, especially the relative proportions of the lower saturated acids.

Characteristic features of these free acid fractions from the products of rancidity which merit some further notice are (i) the presence of 10–20 % of unsaturated, non-volatile residues of low equivalent (210–225) and low iodine number (50–60) which account for about half the unsaturation of the total free acids, (ii) the presence of normal saturated acids from butyric to *n*-nonane-1-carboxylic in similar proportions (averaging about 8 % in each case), (iii) the presence of *n*-octane-1-carboxylic and dibasic (mainly azelaic) acids, (iv) the presence of about 10 % of palmitic acid, and (v) the absence of oleic acid in any great quantity, and the presence of fragments of lower unsaturated acids of indefinite structure.

(i) The unsaturated residues are clearly polymers of some kind as evident from their low iodine values and equivalents and their lack of volatility even under extreme conditions. The basis of these residues is evidently some product derived from oleic acid. Farmer (1942) first demonstrated that atmospheric oxidation of the oleic glyceride begins with the formation of a hydroperoxide group, $-\text{CH}(\text{O OH})\text{CH}_2\text{CH}_2-$, which on further oxidation of the double bond forms short-chain fatty acids. In the view of Hilditch (1947*b*) the mechanism tends to proceed in the following sequence: loose attachment of oxygen at an ethenoid linkage, electronic displacements in the system thus produced, leading to the loosening of a hydrogen atom from an adjacent methylene group and the final formation of a hydroperoxide of the type shown above, the double bond having meantime shifted one step along the carbon framework. These hydroperoxides could conceivably polymerize with ease to produce partly unsaturated polymers. Moreover, the small percentage (up to 4) of linoleic glycerides present in ghee probably acts as a starter for the

whole mechanism by the formation of highly reactive conjugated unsaturated products. Polymerization occurring to such a marked extent in a relatively saturated fat does not appear to have been recorded previously.

(ii) and (iii) It was pointed out earlier from a series of observations that the lower saturated acids are probably produced largely from the oxidation of oleic glycerides. The formation of roughly similar amounts of the acids is explicable in terms of the above theory whereby the double bond apparently shifts during autoxidation: one could conceivably continue the process further along the carbon chain, and couple it with lipolysis at any stage, the observed presence, too, of acids probably mono unsaturated, and of low molecular weight (calculated here as nonene, undecene-1-carboxylic acids, etc.) in amounts much larger than in the original butterfats would again be possible on a similar hypothesis, while the production of azelaic acid (clearly much simpler double bond scission occurs) and its higher and lower homologues would follow from hydrolysis of unsaturated intermediate products. The small amount of oleic acid in the free fatty acids hardly calls for explanation since it is even more vulnerable to attack in the free form, if produced, than as a glyceride.

(iv) The presence of 10 % of palmitic acid can hardly be traced to oleic acid, for even though saturation and chain shortening have been suggested in consequence of certain *in vivo* experiments, these are very unlikely in the present instance to have occurred concurrently with preponderating oxidation. Clearly lipolysis of glycerides can occur to a certain extent even in a rendered (sterile) butterfat, probably from subsequent enzymic contamination during storage, but it is apparent from the experiments now recorded that oxidation products predominate in the end products of rancidity.

Finally, the low saponification equivalents of the total free fatty acids (170–200) reveal that a considerable error may be introduced when expressing the free fatty acidity of ghee 'as % oleic acid', as is often the case. It is suggested that either the acid value, which is independent of the nature of the free acidity, be used, or else that a figure of, say, 200 be taken as approximating to the average equivalent of the free acids normally produced during rancidity.

SUMMARY

1 A study has been made of three characteristic sets of ghee (Indian butterfat), comprising about a dozen very similar samples in each, after maintenance in loosely corked bottles at temperatures between 15 and 20° over a period of 3 years.

2 Changes in the analytical constants were strikingly similar—increases in Reichert value, marked increases in Polenske and saponification

values, losses in iodine value, and rather erratic changes in refractive index

3 Marked parallelisms between the alterations of the characteristics for any one sample suggested that these alterations proceed from a single general cause, most probably aerial oxidation. Increases in acidity, in particular, exactly paralleled loss in iodine value for any particular batch of samples, suggesting an essentially oxidative mechanism producing free acidity in butterfat as opposed to a mainly lipolytic one in butter.

4 The mixed free fatty acids from each batch of pooled rancid samples have been extracted thoroughly with ethanol and analyzed for component acids. Important features were the presence of *n*-octanoic acid to the extent of 7–9 mol % (proved by the preparation of its *p*-toluidide derivative, a series of such derivatives from pure fatty acids being also incidentally prepared for reference purposes) and of normal homologous saturated acids from butyric to *n*-nonanoic in roughly similar amounts, the, perhaps adventitious, presence of acetic acid (as shown both by chromatographic and direct fractionation methods), the occurrence of azelaic acid, with traces of unidentified higher and lower homologous dicarboxylic acids, the presence of about 10 % of palmitic acid, the absence of any great quantity of oleic acid, but the presence of fragments of uncharacterized lower unsaturated acids in small amounts, and of non-volatile residues of low equivalent and low iodine value to the extent of 10–20 % (accounting for nearly half of the total unsaturation).

5 Most of the above features are considered to be explicable as resulting from the auto-oxidation, probably as a glyceride, of oleic acid by recently proposed mechanisms. Polymerization also emerges as an important feature of rancidification in these comparatively saturated fats. Glyceride lipolysis also occurs, but to smaller extents than the two effects just enumerated.

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The Use of the Tyrosine Apodecarboxylase of *Streptococcus faecalis* R for the Estimation of Codecarboxylase

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The bacterial L lysine and L tyrosine decarboxylases have been shown to contain a coenzyme known as codecarboxylase, this substance was found to be widely distributed in nature (Gale & Epps, 1944a, b, Epps, 1944). Gale & Epps have used the apoenzyme of the L tyrosine decarboxylase of *Streptococcus faecalis* for the estimation of codecarboxylase, the rate of decarboxylation of tyrosine was a function of the amount of codecarboxylase added, the apoen-

zyme was prepared by the dissociation of the holoenzyme. A method of estimating codecarboxylase by means of tyrosine apodecarboxylase has also been described by Umbreit, Bellamy & Gunsalus (1945). They used, as source of apoenzyme, suspensions of freeze dried cells of *Strep faecalis* R, which had been grown in a vitamin B₆-free medium. Such preparations also contained an enzyme system which formed codecarboxylase from pyridoxal and adenosinetri-

phosphoric acid (ATP). Therefore, in the presence of excess ATP the rate of decarboxylation of tyrosine was a function of the amount of pyridoxal added.

The present paper describes experiments in which acetone dried preparations of vitamin B₆-free cells of *Strep faecalis* R were used for the estimation of codecarboxylase in tissues. In the course of this work, some new observations were made on the coenzyme-apoenzyme relationship of the tyrosine decarboxylase system of *Strep faecalis* R.

METHODS

The methods used were similar to those described by Umbreit *et al.* (1945). The preparation of intact and acetone dried cells of *Strep faecalis* R has been described elsewhere (Sloane Stanley, 1949). Cells grown in a vitamin B₆ free medium contained tyrosine apodecarboxylase but no codecarboxylase, they will be called deficient cells. Cells grown in the same medium but in the presence of pyridoxal contained the enzyme saturated with codecarboxylase, they will be referred to as complete cells.

The manometric procedure was similar to that already described (Sloane Stanley, 1949). Unless otherwise stated, the total fluid volume in each flask was 3.0 ml. The side bulbs contained 0.4 ml. of a 0.04M L tyrosine suspension. The contents of the main compartments differed with the type of cell preparation used, as shown in Table 1. The cell preparations

Table 1. Contents of main compartments of the manometer flasks

(Total vol. of fluid 2.6 ml.)

(a)	(b)	(c)
Dried deficient cells	Dried complete cells	Intact deficient cells
0.2 ml. 0.05M Acetate buffer (pH 5.5)	0.2 ml. 0.05M Acetate buffer (pH 5.5)	1.0 ml. 0.075M-Phthalate buffer (pH 5.0)
1.0 mg. Cells	10 mg. Cells	0.5 ml. Cell suspension, from 3 ml. medium
Source of codecarboxylase	—	Pyridoxal

were always added last. The bath temperature was 28.5°. In experiments with acetone dried deficient cells and pyridoxal, 0.1 ml. of a sulphate free solution of ATP, containing 1 mg. of the free acid, was added to each flask. The calcium pyridoxal 5 phosphate used in some of the experiments was kindly given by Dr K. Folkers, this substance will also be called synthetic codecarboxylase. For the experiments with acetone dried cells, the gas phase was N₂, because it was found that the rate of decarboxylation of tyrosine was 25% lower in air than in N₂. On the other hand, air was used with intact cells as the rate of the reaction was the same in air as in N₂. The course of the reaction was followed by taking readings every 5 min. In some experiments the rate was low in the first few minutes after tipping, but subsequently increased and became steady. In every experiment the steady rate of evolution of CO₂ was measured and assumed to represent the true enzymic activity of the system, it was expressed in μ l

of CO₂/hr, and is represented by V . The maximum value of V obtained with any given preparation of deficient cells, in the presence of enough codecarboxylase to saturate the apoenzyme, is given as V_{\max} (Umbreit *et al.* 1945), the value of V_{\max} was assumed to be a measure of the concentration of apodecarboxylase in the preparation.

RESULTS

Estimation of codecarboxylase

Acetone-dried deficient cells did not decarboxylate tyrosine at all in the absence of any source of codecarboxylase nor did the addition of ATP to these preparations, in the absence of pyridoxal, cause any decarboxylation. Aqueous suspensions of these cells, stored in the refrigerator, retained their full tyrosine apodecarboxylase content for at least 24 hr.

Preparation of tissue suspensions. At first, the procedure described by Bellamy, Gunsalus & Umbreit (1945) was used. A weighed piece of tissue was homogenized in 0.1N NaOH, the mixture was heated for 5 min. in boiling water and stored in the refrigerator. Before each estimation some of the homogenate was acidified with N HCl to pH approx. 5.5. Samples of the resulting suspension were then added to manometer flasks, as shown in Table 1 (a). Under these conditions V was approximately proportional to the volume of tissue suspension used, up to a value about 60% of V_{\max} . The relation between V and the amounts of tissue added was similar to that illustrated in Fig. 2, curve A.

It was found that the codecarboxylase in homogenates of tissue in 0.1N NaOH was not stable, for instance, in one experiment, a homogenate of rat liver had lost 50% of its original codecarboxylase activity after 18 hr. storage in the refrigerator. In borate buffers, however, the codecarboxylase activities of tissue homogenates were found to be more stable (Table 2). In another, similar experiment, a rat-liver

Table 2. Effect of pH on stability of codecarboxylase in rat liver homogenates

(Contents of manometer flasks as in Table 1 (a), suspension equivalent to 0.5 mg. of rat liver (homogenized in distilled water, and samples heated in the solutions below, for 4 min. at 100°) added to each flask as source of codecarboxylase.)

Solution used for preparation of homogenate (final conc., 0.1M)	Values of V (μ l. CO ₂ /hr) after storage in refrigerator for		
	0 hr	24 hr	72 hr
Borate (pH 8.0)	120	130	100
Borate (pH 9.0)	110	100	90
Borate (pH 10.0)	110	110	80
NaOH	110	80	40

homogenate in 0.01N NaOH lost its codecarboxylase activity even more rapidly. In both these experiments, the loss of activity was least in the borate buffers of pH 8 and 9, in all subsequent assays of codecarboxylase, therefore, the following method was adopted.

A weighed piece of tissue was homogenized in distilled water and 4 ml. of 0.25M borate buffer (pH 8.5) were added to each 6 ml. of homogenate, the mixture was heated for 4 min. in boiling water and stored in the refrigerator. Before use,

a sample of this mixture was diluted with an equal volume of M acetate buffer of pH 5.5, the resulting suspension was diluted with a suitable volume of distilled water. The pH of suspensions thus prepared was found to be 5.5 when measured with the glass electrode. The 0.25M-borate buffers used in these experiments were prepared from H_3BO_3 and NaOH in the proportions given by Cole (1933), but without KCl.

The codecarboxylase in these borate-buffered homogenates was found to be stable. For instance, in one experiment, the equivalent of 1 mg of rat liver gave the following values of V immediately after preparation, 140, after 120 hr in the refrigerator, 150.

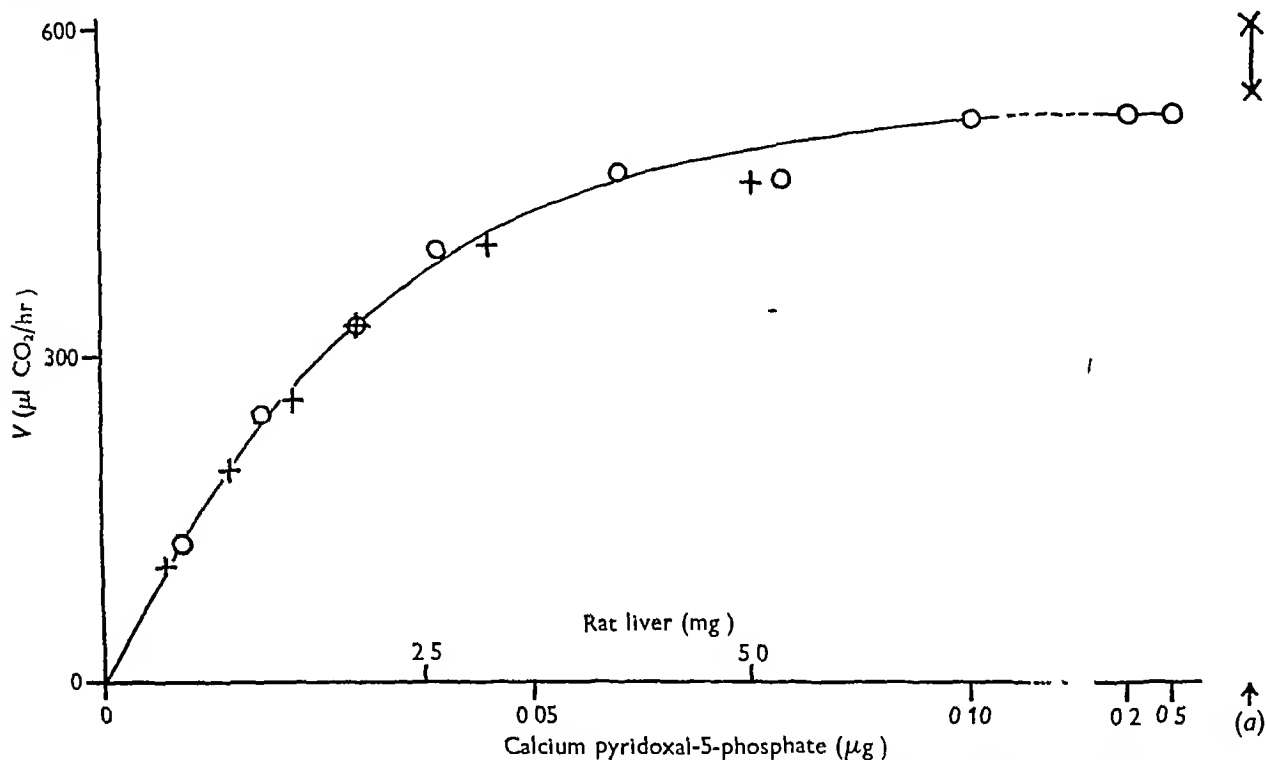


Fig 1 Rates of decarboxylation of tyrosine by acetone dried vitamin B₆ free cells of *Strep faecalis* R, with varying amounts of natural and synthetic codecarboxylase +, rat liver suspension, O, calcium pyridoxal 5 phosphate, (a), x, pyridoxal (10 μg) with ATP (1 mg)

Synthetic codecarboxylase was found to be unstable in distilled water, however, solutions in borate buffer, which had initially the same codecarboxylase activities as solutions in distilled water, were more stable. For instance, a solution of calcium pyridoxal-5-phosphate (0.1 μg/ml) in distilled water lost 60% of its activity in 48 hr at -2° , but a similar solution (0.16 μg/ml) in 0.1M-borate buffer (pH 8.5) kept its full activity for 100 hr at 0° . All subsequent experiments with synthetic codecarboxylase were therefore done with solutions in borate buffer.

Estimation of codecarboxylase with calcium pyridoxal 5 phosphate as standard. The experiments illustrated in Fig 1 showed that with our prepara-

tions of acetone dried deficient cells synthetic codecarboxylase was a suitable standard for the estimation of codecarboxylase in tissues. In these experiments, the amount of apoenzyme was kept constant and varying amounts, either of rat liver (crosses, Fig 1) or of calcium pyridoxal-5 phosphate (circles, Fig 1) were added. The rate of decarboxylation was the same when either 2 mg of rat liver or 0.03 μg of calcium pyridoxal-5-phosphate were added, Fig 1 has, therefore, been drawn so that these two points coincide. It will be seen that all the points for other values of V lie on or very near the same curve, irrespective of whether the source of codecarboxylase was rat liver or calcium pyridoxal-5-phosphate.

As a result of these experiments the following procedure was adopted for the estimation of codecarboxylase in tissues. Determinations were carried out with at least two different volumes of tissue suspension, so chosen that the resulting values of V were approximately proportional to the amounts of tissue used, the equivalent quantities of calcium pyridoxal-5-phosphate were found from the calibration curve of the sample of cells used (e.g. Fig 1), and the mean of the results was taken as the concentration of codecarboxylase in the suspension.

Table 3 gives the results of the assay of three different rat livers, these results were obtained with the sample of cells for which the calibration curve is given in Fig 1. The maximum enzymic activity

(V_{max}) of this preparation did not change in six weeks, but others have been less stable. With the small amounts of codecarboxylase used in these assays, however, small decreases in the tyrosine apodecarboxylase content of a sample of cells are of little importance. For instance, in an experiment with 1 mg of rat liver, V was 150 when 1 mg of cells was used, with 2 mg of cells, V was 180, therefore, doubling the amount of apoenzyme had only increased V by 20 %. With a constant quantity of apoenzyme, V depends on the amount rather than on the concentration of codecarboxylase added. In an experiment with 1 mg of cells and 0.02 μ g of calcium pyridoxal-5 phosphate, V was 240 when the volume of liquid in the manometer flask was 3 ml, when the concentration of codecarboxylase was doubled by reducing the volume to 1.5 ml, V increased by only 8 %, to 260. These results suggest that nearly all the added codecarboxylase combined with the apoenzyme.

Table 3 Estimation of codecarboxylase in rat liver, with acetone-dried deficient cells calibrated with calcium pyridoxal-5 phosphate

Animal used as source of liver*	Amount of liver (mg)	V (μ l CO ₂ /hr)	Codecarboxylase content of liver (m μ g calcium pyridoxal 5 phosphate/mg)
Deficient in vitamin B ₆	0.5	80	11.0
	1.0	150	11.0
			Mean 11.0
Control receiving vitamin B ₆	0.5	110	16.0
	1.0	195	15.0
			Mean 15.5
Control receiving vitamin B ₆	0.5	130	19.0
	1.0	210	16.0
			Mean 17.5

* See Blaschko, Carter, O'Brien & Sloane Stanley (1948)

Estimation of codecarboxylase with pyridoxal as standard. Before synthetic codecarboxylase became available, assays were made using pyridoxal, with excess ATP as suggested by Umbreit *et al* (1945). The relation between V and amounts of pyridoxal added to the acetone dried deficient cells in the presence of ATP was similar to that reported by Umbreit *et al* for freeze dried cells, but difficulties were encountered when attempts were made to express the codecarboxylase activities of tissues in terms of pyridoxal, for two reasons. First, the curves obtained by plotting V against amounts of tissue suspension added to the cells could not be superimposed on those obtained with pyridoxal in the presence of ATP. It was found that V was nearly proportional to the volume of tissue suspension added up to a value of about 60 % of V_{max} , whereas V was proportional to the amount of pyridoxal

added up to a value of only 25 % of V_{max} , yet the values of V_{max} obtained with tissue suspension and with pyridoxal were almost identical (Fig. 2). Secondly, the relation between V and the amount of pyridoxal added to a given preparation of cells was not constant, but varied from one experiment to another, the result of the experiment described below suggests that this may have been due to the time taken for the phosphorylation of added pyridoxal. In this experiment, the manometer flasks were shaken in the bath for varying times before the addition of the tyrosine to the main compartments. With 0.05 μ g of pyridoxal, V increased more than threefold when the time of equilibration was increased from 10 to 130 min, with 10 μ g of pyridoxal, the increase in V was less than 25 %.

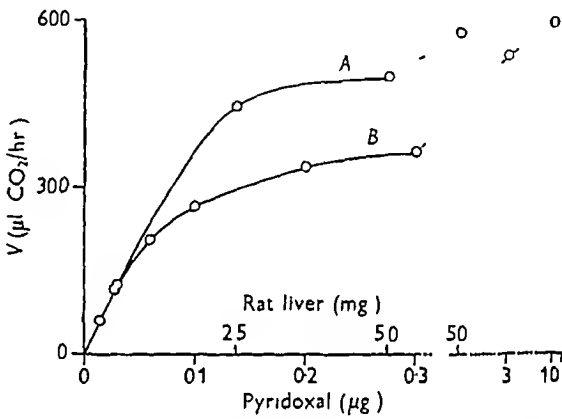


Fig. 2 Rates of decarboxylation of tyrosine by acetone dried vitamin B₆ free cells of *Strep faecalis* R, with varying amounts of rat liver suspension (curve A) and of pyridoxal in the presence of 1 mg ATP (curve B) as sources of codecarboxylase

Synthetic pyridoxal acetal 3-phosphate was tested as a possible source of codecarboxylase, using 1 mg of acetone dried cells. With 20 μ g of pyridoxal acetal-3-phosphate, V was about 30, with 20 μ g of this substance plus 1 mg of ATP, V was about 60, with 0.01 μ g of pyridoxal plus 1 mg of ATP, V was 120, the value of V_{max} for this sample of cells, determined with 10 μ g of pyridoxal plus ATP, was 600. These results do not support the view that pyridoxal acetal-3 phosphate has codecarboxylase activity, they suggest that the sample used may have contained a trace of codecarboxylase and of pyridoxal.

Properties of the tyrosine decarboxylase system

(a) *Acetone dried deficient cells*

Effect of sulphate. It was found that sulphate inhibited the decarboxylation of tyrosine by preparations of acetone dried deficient cells in the presence of codecarboxylase (Table 4). The inhibition increased with increasing sulphate concentration,

when the amount of synthetic codecarboxylase added was kept constant and was almost enough to saturate the apoenzyme. When the concentration of sulphate was kept constant, the inhibition was most marked with small amounts of codecarboxylase, but very nearly disappeared with large

Table 4 *Effect of sulphate concentration on the decarboxylation of tyrosine by acetone-dried deficient cells*

(Source of codecarboxylase 0.07 μ g calcium pyridoxal-5-phosphate)

Concentration of Na_2SO_4 (M)	V (μ l CO_2 /hr)	Inhibition (%)
0	565	—
3.3×10^{-4}	535	5
1×10^{-3}	460	19
3.3×10^{-3}	300	47
1×10^{-2}	190	66
3.3×10^{-2}	100	82

Table 5 *Effect of varying the amount of codecarboxylase on the inhibition by sulphate of the decarboxylation of tyrosine by acetone dried deficient cells*

(Concentration of Na_2SO_4 , 10^{-2} M)

Calcium pyridoxal 5-phosphate added (μ g)	V without sulphate (μ l CO_2 /hr)	V with sulphate (μ l CO_2 /hr)	Inhibition (%)
0.025	285	75	74
0.07	520	170	67
0.2	630	375	40
0.7	645	590	8.5
7.0	715	655	8.5

amounts of codecarboxylase (Table 5). The degree of inhibition by sulphate depended on the order in which sulphate and synthetic codecarboxylase were added to the apoenzyme preparation (Table 6). In

Table 6 *Inhibition by sulphate: effect of varying order of addition of constituents of system*

(Main compartments of manometer flasks contained acetone dried deficient cells and acetate buffer, as in Table 1(a). Source of codecarboxylase 0.025 μ g calcium pyridoxal 5-phosphate (added in 0.25 ml). Concentration of sulphate 10^{-2} M, added as 0.3 ml 0.1 M- Na_2SO_4 . Codecarboxylase in main compartment.)

Manometer no	1	2	3
Sulphate	Not added	In main compartment	In side bulb
V (μ l CO_2 /hr)	285	75	265

Table 8 *Effects of phthalate and phosphate with acetone dried deficient cells, and pyridoxal and ATP*

Pyridoxal added (μ g)	Salt added	V with salt (μ l CO_2 /hr)	V without salt (μ l CO_2 /hr)	Inhibition (%)
10	2.5×10^{-2} M Phthalate buffer (pH 5.5)	180	330	45
0.1	3.3×10^{-2} M-Phthalate buffer (pH 5.5)	20	170	88
0.1	3.3×10^{-2} M KH_2PO_4	100	170	41

the flask in which sulphate was added from the side bulb together with the tyrosine at the moment of tipping, the codecarboxylase had been in contact with the dried cell preparation for about 30 min. Under these conditions the addition of sulphate had an almost negligible effect.

Sulphate also inhibited the reaction in experiments in which pyridoxal and ATP were used as source of codecarboxylase. For instance, in an experiment in which 10 μ g of pyridoxal were added, V was 500 in the absence of sulphate, in the presence of 0.033 M-sodium sulphate, V was 110. In another similar experiment the corresponding figures were without sulphate, 580, with 0.0033 M-sodium sulphate, 390.

Other salts. Tables 7 and 8 give some results obtained with other substances which were found to inhibit the reaction when acetone dried deficient cells were used. The experiments of Table 7 were done with synthetic codecarboxylase, those of

Table 7 *Inhibition of tyrosine decarboxylase system in acetone-dried deficient cells by phthalate, phosphate, and pyrophosphate*

(Source of codecarboxylase 0.07 μ g calcium pyridoxal 5-phosphate)

Salt added	V with salt (μ l CO_2 /hr)	V without salt (μ l CO_2 /hr)	Inhibition (%)
10^{-2} M phthalate buffer (pH 5.5)	30	600	95
3.3×10^{-3} M KH_2PO_4	290	600	52
3.3×10^{-3} M KH_2PO_4	480	475	0
3.3×10^{-2} M $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$	230	455	50
3.3×10^{-2} M $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$	490	475	0

Table 8 with pyridoxal and ATP. It will be seen that phthalate was a stronger inhibitor than sulphate, whereas phosphate and pyrophosphate had a much weaker action. Some chlorides were also tested. NaCl, KCl and BaCl_2 , each in a final concentration of 0.033 M, did not affect the rate of decarboxylation of tyrosine by 1 mg of acetone dried deficient cells in the presence of 0.07 μ g of calcium pyridoxal-5-phosphate.

(b) *Acetone-dried complete cells*

With complete cells, the inhibitory effects of the salts tested as described above were either absent or less marked. There was no inhibition with sulphate, phosphate, or pyrophosphate. With phthalate the

degree of inhibition was only 22 %. All these substances were tested in a final concentration of 0.033M.

(c) Intact cells

With intact deficient cells, substitution of 0.067M-acetate buffer (pH 5.0) for the phthalate buffer normally used had no effect on the value of V . Using intact deficient cells in the same acetate buffer, the value of V was not affected by the addition of 0.033M-sulphate, phosphate or pyrophosphate.

It was found that codecarboxylase did not restore the complete tyrosine decarboxylase system in intact deficient cells. Experiments were done with both synthetic and natural codecarboxylase. For instance, the addition of 3.7 μ g of calcium pyridoxal-5-phosphate to a washed suspension of intact deficient cells did not cause any significant decarboxylation of tyrosine ($V > 8$), yet V equalled 430 in the same experiment when 10 μ g of pyridoxal were added. In an experiment with rat liver suspension as source of codecarboxylase, the equivalent of 110 mg of rat liver was added to a sample of intact deficient cells, thus did not cause any decarboxylation of tyrosine ($V = 0$), as compared with $V = 320$ when 10 μ g of pyridoxal were added. The amount of codecarboxylase in 110 mg of rat liver is equivalent to about 1.65 μ g of calcium pyridoxal-5-phosphate, which was more than enough to saturate the apoenzyme in 1 mg of an active sample of acetone-dried deficient cells (Fig. 1).

DISCUSSION

Difficulties were encountered in the attempt to estimate codecarboxylase in terms of pyridoxal, using acetone dried preparations of *Strep faecalis* R. The experiments described above suggest that this was mainly due to an impairment in such cells of the system which forms codecarboxylase from pyridoxal and ATP. The difference between our results and those of Umbreit *et al.* (1945) is probably explained by our use of acetone dried instead of freeze-dried cells. It has been shown, however, that acetone-dried preparations of vitamin B₆ free cells (deficient cells) permit the estimation of tissue codecarboxylase in terms of the synthetic substance, with a probable error of $\pm 10\%$. Our observations support the findings of Gunsalus & Umbreit (1947) that synthetic pyridoxal acetal-3-phosphate, which was claimed by Karrer & Viscontini (1946) to be an active form of codecarboxylase, has in fact little or no codecarboxylase activity.

The finding that codecarboxylase is unstable in distilled water agrees with observations of Umbreit *et al.* (1945). Borate buffer (0.1M, pH 8.5) has proved useful in preserving the codecarboxylase activities of solutions of both natural and synthetic codecarboxylase. This effect of borate is of interest since there

is evidence for the formation of a stable complex between borate and pyridoxine (Scudi, Bastedo & Webb, 1940).

The experiments on the inhibitory action of sulphate on the tyrosine decarboxylase system suggest that sulphate has no effect on the complete tyrosine decarboxylase, but that it acts by preventing the combination of codecarboxylase with tyrosine apodecarboxylase. This is supported by the observation that the inhibition occurred only with acetone dried deficient cells whatever the source of codecarboxylase used, while sulphate had no effect on acetone-dried complete cells. The finding that, with acetone dried deficient cells, the inhibitory effect of sulphate was most marked with small amounts of codecarboxylase but was small with large amounts of codecarboxylase, suggests that sulphate competes with codecarboxylase for the apoenzyme. The finding of sulphate inhibition is of practical importance in the estimation of codecarboxylase in terms of pyridoxal in the presence of ATP, since it makes it necessary to ensure that the ATP solutions used are free of sulphate.

Our observations on the inhibitory effects of phosphate and phthalate are in agreement with those of Epps (1944). With intact deficient cells, no inhibitory effect of sulphate, phthalate, phosphate or pyrophosphate on the decarboxylation of tyrosine in the presence of pyridoxal was observed.

It has been reported that in growing cells of *Strep faecalis* R, synthetic codecarboxylase has no vitamin B₆ activity (Rabinowitz & Snell, 1947). This is of interest in view of the inability of intact deficient cells to decarboxylate tyrosine in the presence of large amounts of codecarboxylase. It seems that in the cells of *Strep faecalis* R the apoenzyme is inaccessible to added codecarboxylase. Our results suggest that intact deficient cells of *Strep faecalis* R could be used to detect pyridoxal in the presence of codecarboxylase. The absence of any decarboxylation of tyrosine when rat liver suspensions were used with intact deficient cells shows that the pyridoxal content of the suspensions was negligible.

SUMMARY

1. The use of acetone dried cells of *Streptococcus faecalis* R, grown in a vitamin B₆ free medium, as a source of tyrosine apodecarboxylase for the estimation of codecarboxylase, has been investigated.

2. Satisfactory assays of codecarboxylase in animal tissues can be made with such preparations, using calcium pyridoxal-5-phosphate (synthetic codecarboxylase) as standard.

3. Solutions of both synthetic and natural codecarboxylase in 0.1M-borate buffer (pH 8.5) keep their full initial codecarboxylase activities for several days in the cold.

4 With acetone dried deficient cell preparations, pyridoxal in the presence of ATP is not a satisfactory standard. This is probably due to the low activity of the pyridoxal-phosphorylating system in such preparations.

5 Sulphate, phthalate, phosphate and pyrophosphate inhibit the decarboxylation of tyrosine by acetone-dried deficient cells in the presence of small amounts of codecarboxylase, the inhibition by sulphate is reduced or abolished by an increase in the amount of codecarboxylase added. These substances do not inhibit the decarboxylation of tyrosine by intact deficient cells in the presence of pyridoxal, with acetone dried preparations of cells grown in a medium containing excess pyridoxal they have little or no inhibitory effect. The action of these salts is, therefore, interpreted not as an inhibition

of the holoenzyme, but as an interference with the reaction between codecarboxylase and the apoenzyme.

6 Vitamin B₆ free intact cells of the organism do not decarboxylate tyrosine in the presence of either synthetic or natural codecarboxylase.

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A Quantitative Study of Complex Formation in Heated Protein Mixtures

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When proteins are heated together under certain conditions, the heat denatured particles of different proteins can combine to form complex aggregates. Previous work on such complexes has been solely qualitative. It has shown that complexes are formed (Kleczkowski, 1945) and that they can differ from their components in solubility and precipitability by salts (Kleczkowski, 1941a), electrophoretic mobility (Van der Scheer, Wyckoff & Clarke, 1941), isoelectric point (Kleczkowski, 1946) and serological behaviour (Kleczkowski, 1941b, Bawden & Kleczkowski, 1941, 1942a, b), but there has been no quantitative work on their formation or properties. The present paper describes (1) the effect of varying the concentrations and ratios of the components in heated mixtures on the constitution of the resulting complexes, and (2), the effect of variations in the constitution of the complexes on their solubility and serological behaviour.

MATERIALS AND METHODS

The proteins used were tobacco mosaic virus (TMV), tomato bushy stunt virus (BSV) and human serum albumin. The viruses were purified by the methods described by Bawden & Pirie (1943). For convenience the heat-denatured viruses will be referred to as viruses, although they are non-infective and heat denatured TMV has also lost its serological activity and its nucleic acid. The albumin was a crude unfractionated preparation from human serum. After removal of the globulin by half saturation with $(\text{NH}_4)_2\text{SO}_4$ and filtration, the filtrate was saturated with the salt. The precipitated albumin was collected by filtration, dissolved in water and dialyzed against water. Toluene was added as an antiseptic.

In all experiments designed to test the effect of heat, the protein solutions were made with M/15 phosphate buffer at pH 6.8 and heated in thin walled test tubes immersed in a water bath. Except when otherwise stated, the time of heating was 10 min and the temperature of the water bath 83°. Under these conditions, when heated alone, both viruses

coagulate, whereas when they are heated in the presence of sufficient albumin, no coagulum separates

The complexes formed between albumin and each of the viruses were isolated by precipitation with $(\text{NH}_4)_2\text{SO}_4$, which was used at concentrations insufficient to precipitate albumin after this protein had been heated alone. The precipitates were sedimented by centrifuging for 10 min at 6000 rev/min and washed 3 times with 5% (w/v) trichloroacetic acid. Their N, P and carbohydrate contents were estimated, and the relative colour intensity given by them in the May & Rose (1922) test for tryptophan was determined. From these results the total protein and the ratio of the constituent proteins in the complexes were obtained. N was estimated by the micro Kjeldahl method, and translated into protein by multiplication by 6.4. P and carbohydrate were estimated colorimetrically (Kleczkowski, 1946). The colorimetric test for tryptophan differed in some respects from Bates's (1937) modification of the May & Rose (1922) test. The reagent (6 ml) was added to 0.4 ml of a protein solution or suspension, the mixture incubated for 5 min at 75° and then cooled, the intensities of the blue colours formed by different protein preparations were compared in a colorimeter of Duhosq type. (The reagent consisted of 25 ml of conc HCl + 0.5 ml of 5% (w/v) *p* dimethylaminobenzaldehyde in 10% (w/v) H_2SO_4 + 0.2 ml of 1% (w/v) NaNO_3 . The constituents were freshly mixed for each test.)

RESULTS

Aggregation of the albumin heated alone

As a preliminary to using precipitation with ammonium sulphate for isolating the complexes, the effect of heating on the albumin alone was studied. It was found that the precipitability of heated albumin by the salt depends on several factors such as the temperature and the duration of heating, the concentration of albumin during heating, the concentration of albumin after heating when the salt is added, and the lapse of time between adding the salt and centrifuging. For the purpose of this work variation in the albumin concentration during heating was the most important of these factors, and its effect was studied in detail. Fig. 1 shows the effects of heating 1, 0.5 and 0.25% albumin solutions. After cooling, the 1 and 0.5% albumin solutions were diluted to four times and twice their volumes respectively with $\text{M}/15$ phosphate buffer at pH 6.8 to bring all the solutions to the same protein concentration. Different amounts of saturated ammonium sulphate solution were added to different samples from each solution. The mixtures were kept for 15 hr at room temperature, centrifuged for 10 min at 6000 rev/min and the amounts of sedimented protein were estimated. These, expressed as percentages of total albumin, are plotted in Fig. 1 against concentrations of ammonium sulphate, expressed as percentages of saturation.

It will be seen that the higher the concentration of the albumin during heating, the lower was the minimum concentration of the salt necessary to

produce precipitation and the greater was the amount precipitable by salt concentrations between 31 and 50% saturation. On the other hand, at 60% saturation, which precipitated only about 4% of the unheated albumin, about 85% of the total albumin was precipitated from all three heated solutions. It seems that the proportion of albumin denatured by heat did not depend appreciably on the albumin concentration during heating. The 85% of the total albumin which was rendered precipitable by 60% saturated ammonium sulphate probably consisted of fractions that denature rapidly at 83°, and the remaining 15% (probably mainly glycoprotein) of fractions with lower denaturation rates or with solubilities not greatly affected by heat.

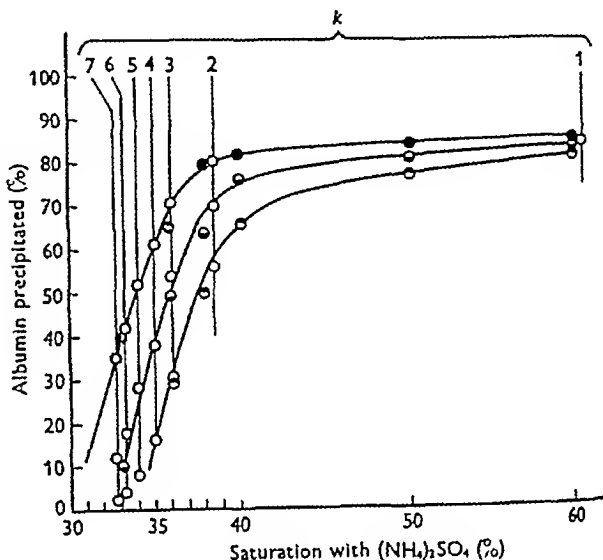


Fig. 1 Percentages of heated albumin precipitated by different concentrations of ammonium sulphate. ●, Albumin heated in 1% solution, ◐, in 0.5% solution, ○, in 0.25% solution, ○, values of $0.85p$ (p being obtained from equation (1), for significance of k see text).

Pedersen (1931) has shown that when albumin solutions, ultracentrifugally homogeneous before heating, are heated under conditions in which coagulation does not occur, they contain after heating, components with different sedimentation constants. From theoretical considerations of the kinetics of aggregation it is to be expected that the higher the concentration of a heated albumin solution, the greater will be the extent of aggregation. It seems likely that precipitability with ammonium sulphate at concentrations lower than 60%, at which all the denatured albumin separates, depends on the extent to which the denatured protein molecules have aggregated. Supporting evidence for this assumption was obtained from the results of an experiment in which the three heated albumin solutions were adjusted to a concentration

of 0.25% and centrifuged for 30 min at 40,000 rev/min in an air-driven angle centrifuge. The pellets produced contained 60, 40 and 20% of the total protein from the solutions heated at 1, 0.5 and 0.25% albumin respectively, whereas no pellet was obtained from an unheated solution of the albumin. These proportions corresponded approximately with those of the amounts precipitated from the three solutions by 35% saturated ammonium sulphate (Fig 1).

From Smoluchowski's (1916, 1918) theory of aggregation in monodisperse colloidal solutions with approximately spherical particles, the formula (1) is derived for the proportion (p) of material composed of k and more primary particles,

$$p = \sum_{i=k}^{\infty} \frac{i\alpha^{i-1}}{(1+\alpha)^{i+1}} = \left(\frac{\alpha}{1+\alpha} \right)^k \left(\frac{k}{\alpha} + 1 \right), \quad (1)$$

where $\alpha = 8\epsilon\pi R D v_0 t$, and R is the radius of the primary particle (in cm), D is its diffusion constant (in cm²/sec), v_0 is the original number of primary particles in unit volume (ml), t is the length of time of aggregation (in sec) and ϵ is the fraction of collisions resulting in permanent combination.

The results shown in Fig 1 fit formula (1) if three assumptions are made: first, that the 85% of albumin denatured rapidly, so that during most of the 10 min heating at 83° only aggregation was occurring; secondly, that all the heat denatured albumin molecules were of approximately the same size and equally likely to aggregate; thirdly, that the amounts of albumin precipitated from the three heated solutions by a given concentration of ammonium sulphate were at least roughly equal to the amounts of albumin which existed in the form of aggregates composed of at least k primary particles. Different values of k would then correspond with different concentrations of the salt. This can be assumed in spite of the fact that, whereas k takes only integral values, the amount of precipitated albumin is a continuous function of the concentration of ammonium sulphate.

The value of ϵ will be expected to be low because heat denatured albumin molecules remain charged at pH 6.8. All the values determining α , except v_0 , are constant, so that the value of α is proportional to the concentration of heated albumin solutions.

The value of α for one albumin concentration was so chosen that when, for one chosen value of k , the value of p was fitted to the curve in Fig 1, the value of p for another albumin concentration fitted to the corresponding point of the other curve. Thus the values taken for α were 0.7, 1.4 and 2.8 for 0.25, 0.5 and 1% albumin solutions respectively. It will be seen from Fig 1 that when the values of p , corresponding to different values of k , were made to fit to the curve for one of the albumin concentrations, the other two values fitted corresponding points

on the other two curves through the whole range.

From the results of fitting the formula (1) to the curves in Fig 1 it can be concluded that only about half of the material was in the form of aggregates composed of at least five, three and two primary particles in the heated solutions containing 1, 0.5 and 0.25% albumin respectively, and in the 0.25% albumin solution the proportion of material in aggregates containing seven or more primary particles was negligible. The aggregation did not, therefore, progress very far. It can also be concluded that, because the value of ϵ is of the order of 10^{-8} , only one out of about 10^8 collisions resulted in permanent combination. (There are reasons to suppose that the value of ϵ decreases rapidly with decreasing temperature.) So many assumptions are involved, however, that these conclusions can only be considered as tentative.

Estimations of the ratios of the constituents in the complexes

Three solutions were heated: *A*, 0.042% TMV and 0.25% albumin; *B*, 0.042% BSV and 0.25% albumin; *C*, 0.25% albumin. Each was then divided into samples (4 ml) which were mixed with varying amounts of saturated ammonium sulphate solution. The mixtures, and samples containing no ammonium sulphate, were kept for 24 hr at room temperature and then centrifuged for 10 min at 6000 rev/min.

Control solutions of the two viruses heated alone at a concentration of 0.042% coagulated completely and were sedimented by centrifugation without adding ammonium sulphate. The coagula from 4 ml samples from the solutions of TMV and BSV contained 1.6 and 1.7 mg of protein respectively. The coagulum obtained from the solution of TMV was free from nucleic acid, as it contained neither phosphorus nor carbohydrate. The coagulum from the solution of BSV, on the other hand, contained nucleic acid and had 1.3% phosphorus and 6% carbohydrate.

The presence of 0.25% albumin protected TMV from coagulation, and partially protected BSV. When solution *B* was left undisturbed after heating, a coagulum separated, but it formed much more slowly and was much less than in a control solution of the virus heated alone.

Fig 2 shows that some protein was sedimented from solutions *A* and *B* by centrifugation for 10 min at 6000 rev/min in the absence of ammonium sulphate, and progressively more was sedimented with increasing amounts of the salt up to 33% saturation. Nothing was sedimented from the solution of albumin heated alone until the concentration of the salt exceeded 33% saturation.

All the virus was precipitated from solutions *A* and *B* when the ammonium sulphate concentration

reached 33 % saturation. With BSV this was shown by estimation of phosphorus, which constitutes 1.3 % of the virus and only about 0.01 % of the albumin. Thus solution *B* contained 0.022 mg of virus phosphorus/4 ml and only about 0.001 mg of albumin phosphorus/4 ml. The total amount of protein in the precipitate was 4.5 mg with a phosphorus content of 0.023 mg, so that the whole of the virus (1.7 mg) must have been in the precipitate. Thus 2.8 mg of albumin was combined into a complex with the virus.

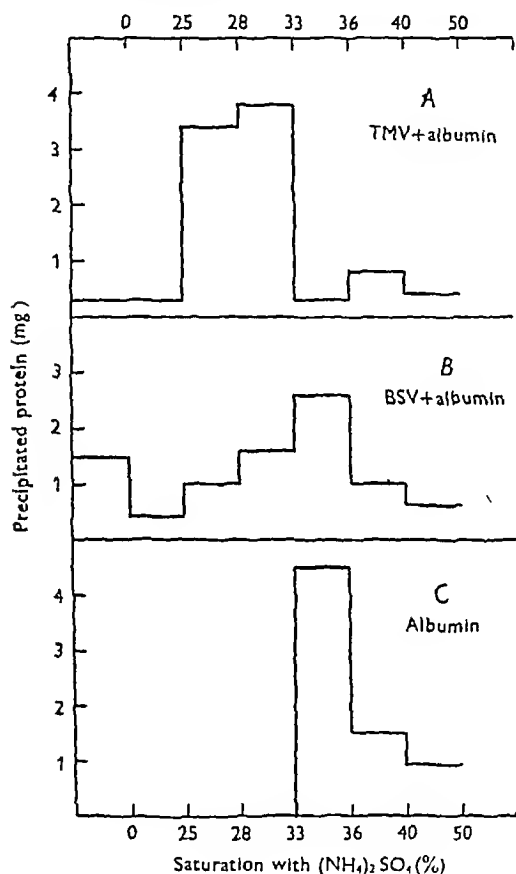


Fig. 2. Precipitating proteins by ammonium sulphate from heated solutions containing TMV, BSV and albumin. The ordinates show the increments in the amounts of precipitated protein corresponding to the increments in the concentration of ammonium sulphate shown by the abscissae. They were obtained by subtracting the amount of protein precipitated at a given concentration of the salt from the amount precipitated at the next higher concentration. *A*, 0.042 % TMV and 0.25 % albumin; *B*, 0.042 % BSV and 0.25 % albumin; *C*, 0.25 % albumin.

The content of TMV in the precipitate obtained from solution *A* by 33 % saturated ammonium sulphate was estimated colorimetrically by the May & Rose (1922) test for tryptophan, in which TMV gives four times the colour given by an equal weight of albumin. The possibility of an albumin fraction, with a chromogenic power different from the average,

participating preferentially in the complex formation could be excluded. It can be seen from Fig. 2 that it is the fraction of heated albumin precipitable between 33 and 36 % saturation with ammonium sulphate which contributed mainly to the complex formation. This fraction was found to have the same chromogenic power as the remaining albumin.

The total amount of protein precipitated by 33 % saturated ammonium sulphate from heated solution *A* was 7.85 mg, which was equivalent in its chromogenic power to 3.2 mg of TVM. Expressing the amount of virus in the precipitate as x , $x + (7.85 - x)/4 = 3.2$, so that $x = 1.65$ mg, i.e. all the virus protein was in the precipitate, and so the complex contained 6.2 mg of albumin.

From these results it follows that the albumin/virus ratios in the complexes were 3.9 and 1.65 with TMV and BSV, respectively. However, the fact that the complexes can be fractionated (Fig. 2) suggests that the ratio of the constituents in single aggregates of the complexes may have varied considerably, so that the ratios should be taken as averages for the two complexes.

The phosphorus content of TMV present in 4 ml of the solution *A* was about 0.009 mg, whereas only a trace of phosphorus (about 0.001 mg) could be detected in the precipitate obtained by 33 % saturation with ammonium sulphate. This can be attributed to the albumin present in the precipitate, as can the 0.06 mg of carbohydrate found there (the carbohydrate content of the albumin is approx. 0.9 %). Thus the heat-denatured protein of the virus, which had formed a complex with heat-denatured albumin, did not contain any appreciable quantity of nucleic acid. The albumin, by forming a complex with the heat-denatured virus, can protect it from coagulation, but not from loss of nucleic acid. Similarly, it does not protect it from the loss of its ability to react with antibodies to the virus.

Bawden & Kleczkowski (1941, 1942*a*) showed that proteins such as BSV, which can be denatured by heat without losing their ability to react with their antibodies, if combined during heating into complexes with enough serum albumin, are not precipitated by their antibodies. They still combine with them, however, and can specifically inhibit the precipitation of unchanged antigens. By contrast, complexes formed during heating between serum albumin and TMV, which loses its serological activity during early stages of heat denaturation, do not inhibit precipitation of unchanged TMV. This has been confirmed with complexes formed during 10 min heating at 80 or 83° in mixtures with albumin/TMV ratios varying from 5:1 to 15:1. No combination between the complexes and virus antibodies occurred to any extent demonstrable either by precipitation or by specific inhibition of precipitation of unchanged virus.

The denaturation rate of TMV does not seem to be influenced by the presence of the albumin. This conflicts with the earlier statement (Bawden & Kleczkowski, 1941) that addition of serum albumin protects TMV from the loss of serological activity and infectivity during heating. The solutions used in the previous work were made in 0.9% sodium chloride and were unbuffered, and unnoticed differences in the pH between virus-albumin mixtures and solutions of the virus alone probably explain the apparent protection. In this work, with all the solutions containing M/15 phosphate buffer at pH 6.8, no differences in serological activities have been found between heated virus-albumin mixtures and solutions of the virus heated alone. At 83 or 80°, denaturation of TMV at pH 6.8 is so rapid that no comparisons can be made, but comparison is possible at 77°. Heating at 77° for varying lengths of time caused equal decreases in the precipitin titres, with an antiserum to TMV, in solutions of the virus alone and in those containing virus and albumin. This was so in spite of the fact that virus-albumin mixtures remained clear, whereas coagula appeared in the solutions of the virus alone.

The effects of varying the ratio of the components in heated mixtures

The complexes were isolated by precipitation with ammonium sulphate used at concentrations insufficient to precipitate control solutions of albumin

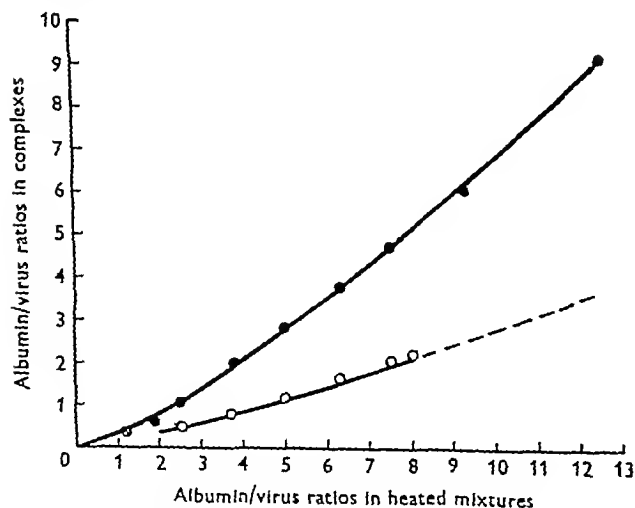


Fig 3 Albumin/virus ratios in complexes formed in heated solutions containing the virus at constant concentration (0.04%) and albumin in varying concentrations, ●, complexes between TMV and albumin, ○, complexes between BSV and albumin

heated alone, and tests were made to ensure that all the virus was in the isolated complex. Fig 3 shows the ratios of albumin to virus in the complexes formed in heated mixtures in which the virus concentration was kept constant at 0.04% and that of the albumin

varied. The method of isolating the complexes worked satisfactorily with the TMV-albumin mixtures over the whole range of the ratios shown. With BSV-albumin mixtures, however, it was satisfactory only until the albumin/virus ratio in the mixture reached 8. At ratios higher than 8 the complexes were not precipitated by ammonium sulphate until the salt concentration was raised to the level at which control albumin solutions also precipitated. Within the range tested, an increase in albumin/virus ratio in the heated mixture corresponded with a slightly more than proportional increase of the ratio in the complex formed. Thus, as the concentration of the albumin increased, so did the proportion of it combined with the virus into the complex (see Fig 4).

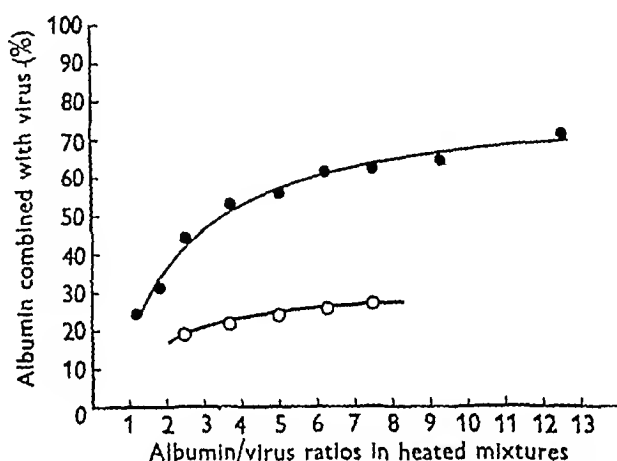


Fig 4 Percentages of albumin combined into complexes with the virus in heated solutions containing the virus at constant concentration (0.04%) and albumin in varying concentrations, ●, TMV, ○, BSV

Figs 3 and 4 also show that under similar conditions the amount of albumin combining with TMV was about 2.4 times that combining with BSV. The same amount of albumin combined with both viruses when the albumin/virus ratio in the heated mixture was about twice as great with BSV as with TMV. Similarly, when the two viruses were in equal concentrations, the minimum concentration of albumin necessary to protect BSV from heat coagulation was about twice as great as that necessary to protect TMV. For example, to protect 0.04% BSV at least 0.3% albumin had to be present, whereas with 0.04% TMV 0.15% albumin was sufficient. Under these conditions, the albumin/virus ratio in both complexes was about 1.8 and this ratio in the complex seems to be the minimum necessary to prevent heat coagulation of either virus.

When the albumin concentration in heated mixtures was kept constant and that of the virus varied, an increase in virus concentration was accompanied by a slightly less than proportional decrease in the albumin/virus ratio in the complex

(Table 1) From this, and from the results shown in Figs 3 and 4, it can be deduced that, if the ratio of the components in heated mixtures is kept constant but the concentration is varied, the albumin/virus ratio in the complex should increase with the increase in the concentration. This was directly shown experimentally as described in the next section.

Table 1 *Effect of variation in virus concentration on the albumin/virus ratio in the complex*

Composition of heated mixtures			Albumin/virus ratios in the heated mixtures	Albumin/virus ratios in the complexes
TMV (%)	BSV (%)	Albumin (%)		
0.04	Nil	0.5	12.5	9.0
0.08	Nil	0.5	6.25	4.6
Nil	0.04	0.5	12.5	3.8*
Nil	0.08	0.5	6.25	2.2

* Value obtained by extrapolation in Fig. 3

The effect of simultaneous variation in the concentrations of both components in heated mixtures

Fig. 5 shows the albumin/TMV ratios in the complexes formed in solutions containing both components at a constant ratio of 6:1 but at varying

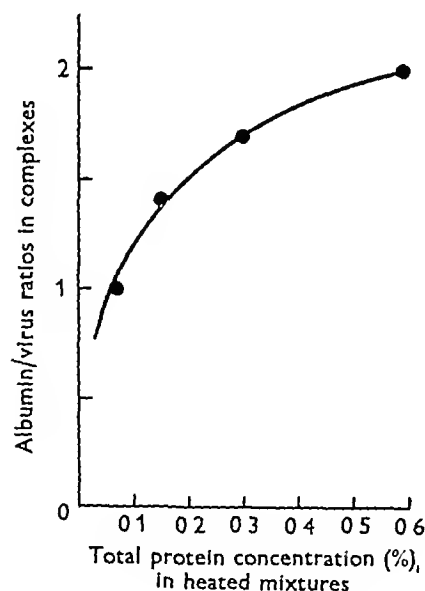


Fig. 5 The effect of total protein concentration in mixtures of albumin with TMV, at a constant ratio of the components (6:1), on the albumin/virus ratios in complexes formed during heating for 10 min. at 80°

concentrations, and heated for 10 min. at 80°. It will be seen that as the total protein concentration increased, so did the albumin/virus ratio in the complex. Within the range tested, when the protein

concentration increased geometrically, the ratio increased arithmetically.

Table 2 shows a comparison of the albumin/virus ratios in complexes formed by albumin with TMV and BSV, when the mixtures were heated at a constant ratio of the components but at two different concentrations. The ratio in the complexes formed with BSV depends on the total protein concentration as it does with TMV, although under the same conditions the ratios with BSV are smaller than those with TMV. Table 2 also shows that the ratios increase with increase in temperature.

Table 2 *Effect of total protein concentration on the ratio of the component proteins in the complexes*

Composition of heated mixtures			Albumin/virus ratios in the complexes	
TMV (%)	BSV (%)	Albumin (%)	10 min at 80°	10 min at 83°
0.04	Nil	0.25	1.7	3.7
0.08	Nil	0.5	2.0	4.6
Nil	0.04	0.25	—	1.6
Nil	0.08	0.5	—	2.0

Changes in the ratio of protein components in complexes, resulting from changes in protein concentration, can account for an apparently paradoxical phenomenon occasionally observed when protein mixtures are heated at different dilutions. Dilution, with ionic strength and pH kept constant, is one method of preventing coagulation of heated protein solutions, as the formation of large aggregates of protein particles is prevented. Occasionally, however, dilution before heating leads to coagulation during the heating. An example is shown in Table 3.

Table 3 *Effect of total protein concentration in mixtures of BSV with albumin on stability of heat denatured virus*

Concentration of constituents in heated mixtures		Appearance of the fluids after heating	Albumin/virus ratio in the complexes
BSV (%)	Albumin (%)		
0.08	0.5	Opalescent but transparent and stable	2.0
0.04	0.25	Small floccules settling slowly	1.6
0.02	0.125	Large floccules settling rapidly	1.2

where an undiluted solution containing 0.08% BSV and 0.5% albumin did not coagulate after heating, but did if diluted twice or four times before heating.

When the albumin/virus ratio in the complex formed during heating was 2.0, the virus, at a concentration of 0.08%, was protected from coagulation. When, because of diluting the mixture before heating, the ratio fell to 1.6 and 1.2, there was not enough albumin combined with the virus to prevent its coagulation, although the virus concentration fell to 0.04 and 0.02%, respectively.

DISCUSSION

This work was limited to complexes formed during heat denaturation between pairs of proteins, one of which when heated alone becomes insoluble while the other remains soluble. The ratio of the amount of the former to that of the latter in heated mixtures was limited to values less than 1. Of the factors

Table 4 *Effect of concentration of heated albumin BSV mixtures on serological properties of the complex*

(No coagulation appeared in any of the heated solutions. After cooling they were diluted (in saline) to bring the virus concentration to the values indicated. Precipitin test: 1 ml of an antiserum to the virus at a dilution of 1/500 was added to 1 ml of antigen solutions at various concentrations, and the mixtures placed in a water bath at 50°. + signs indicate the degree of precipitation, - signs indicate no precipitation where the test for inhibition was not made. Test for inhibition: 0.1 ml of 0.05% solution of unheated virus was added to the tubes where there was no precipitation after 3 hr incubation, + indicates inhibition (no precipitation), 0 indicates no inhibition (precipitation).)

Concentration of constituents in heated mixtures		Albumin/virus ratios in the complexes	Precipitin test Virus concentration (g/100 l)					
BSV (%)	Albumin (%)		10	5	2.5	1.25	0.625	0.312
0.08	1.0	4.4*	+	+	+	+	0	0
0.04	0.5	3.7*	0	0	0	0	0	0
0.02	0.25	3.0	++	+	0	0	0	0
0.01	0.125	2.3	+++	+++	++	+	-	-
0.005	0.0625	1.6*		+++	++	+	±	-
Unheated virus (control)			+++	+++	++	+	±	-

* Values obtained by extrapolation from results of other experiments

The decrease in the albumin/BSV ratio in the complex, resulting from reduced protein concentration, is also reflected in serological reactions of the complex with antibodies to the virus. The results of an experiment described in Table 4 show that when a solution containing 0.08% BSV and 1% albumin was heated, a complex was formed which contained over four times as much albumin as virus. The complex did not precipitate with virus antiserum and it inhibited precipitation of subsequently added unheated virus. When the concentration of the virus albumin mixture was reduced by half before heating, the complex with albumin/virus ratio 3.7 gave no precipitate with the antiserum, and showed no inhibiting power. When the concentration of the virus albumin mixture was still further reduced before heating, the ratio fell still further and, although there was still enough albumin in the complex to prevent coagulation, there was not enough to prevent precipitation with the antiserum to the virus. This agrees with previous work (Kleczkowski, 1941b) which has shown that formation of non-precipitating and inhibiting complexes between antibodies and other serum proteins in heated antisera can be prevented by increasing the dilution of the antisera in saline before heating.

likely to affect the composition and properties of complexes only variations in the concentration and in the ratio of the components in heated mixtures were studied.

The conclusions reached here can be expected to apply in general trends to similar protein systems, i.e. to pairs of proteins, one of which when treated alone becomes insoluble, whereas the other remains soluble, e.g. a serum globulin and a serum albumin. Numerical relationships true of one system are obviously inapplicable to another. For example, these differed in TMV albumin and BSV-albumin systems, where complexes with different ratios of the components were formed under similar conditions. The general trends, however, were similar, for variations in the ratio and in the concentration of the components of the heated mixtures changed the ratios of the components in the complexes similarly in both systems.

A number of other factors, not studied in this work, are likely to influence the composition and properties of complexes formed between pairs of different proteins during heat denaturation. Among these are variations in the pH and in the character and concentration of salts present in heated solutions, it is known that complexes are not formed to any

appreciable extent in salt-free solutions, and that there is an interaction between the pH and the character and concentration of salts (Kleczkowski, 1943)

If the conclusions about the extent of aggregation of the albumin heated alone, deduced from the curves of precipitability by ammonium sulphate with the help of Smoluchowski's theory of aggregation (see Fig 1), are at least approximately true, heat denatured albumin molecules combine with heat-denatured virus particles much faster than among themselves. For example, in the heated 0.25% albumin solution about 60, 30 and 20% of the albumin existed as aggregates of at least two, three and four molecules respectively, and almost none as aggregates of seven or more molecules. By contrast, when mixtures containing 0.25% of the albumin and 0.042% of TMV or BSV were heated under the same conditions, about 60 and 25% of the albumin formed a complex with TMV and BSV respectively. Particles of BSV are approximately spherical and equal in size, and there is no reason to suspect that they break into fragments during heat denaturation. Assuming that the weight of a particle of BSV, in terms of molecular weight, is 7×10^6 , and that of the albumin molecule is 7×10^4 , then the 25% of the albumin would be combined into a complex with BSV with an average of 160 albumin molecules to one virus particle. Similar reasoning cannot be applied to TMV, the particles of which vary in size very considerably and undergo much greater changes during heat denaturation than do BSV particles. It is probable, however, that an average number of albumin molecules/virus particle in the albumin-TMV complex was even higher, perhaps 10 times, than that in the albumin BSV complex. (For example, the ratio of 2000:1 would be obtained, if the average weight of TMV particles in the preparation used were taken as 5×10^7 , and if it were further assumed that this was unaltered by heat denaturation.)

Two reasons can be given for aggregation of albumin molecules proceeding much faster with virus particles than among themselves. First, the proportion of collisions resulting in permanent combination may be higher in those between albumin molecules and virus particles than in those between albumin molecules themselves. Secondly, Wiegner (1911) and Galecki (1912) have shown that, in colloidal solutions containing particles of considerably different sizes, particles of greater sizes function as nuclei of aggregation for particles of smaller sizes. This is so because the probability of a collision between two particles, subjected to Brownian movement, is greater when they are of greatly different sizes than when they are of equal or not greatly

different sizes (see Muller, 1928). The probability of a collision between two particles of the same size is independent of the size, and, when the sizes are not equal, it is increased by the factor

$$f = \frac{(R_1 + R_2)^2}{4R_1R_2},$$

where R_1 and R_2 are the radii (in any units) of the two particles, both assumed to be spherical. Thus, if heat denatured albumin molecules and BSV particles are assumed to be approximately spherical and of the weights given above, the probability of a collision between an albumin molecule and a virus particle would be 1.7 times greater than between two albumin molecules or two virus particles. The probability of a collision of an albumin molecule with an 'average' particle of heat denatured TMV is probably greater than with a particle of BSV.

SUMMARY

1. The ratios of the constituent proteins in complexes formed by human-serum albumin with tobacco mosaic virus and with tomato bushy stunt virus during heat denaturation in solutions containing M/15 phosphate buffer pH 6.8, were studied.

2. When heated alone both viruses coagulate, whereas the albumin does not, although it aggregates increasingly with increasing concentration. When the albumin/virus ratio in the complex is 1.8 or higher, the complex forms a stable solution. All the virus and only part of the albumin participate in the formation of such a complex.

3. Heat-denatured tobacco mosaic virus, combined with albumin, contains no nucleic acid and has no serological activity, whereas the complex of bushy stunt virus retains both. When the albumin/bushy stunt virus ratio in the complex is over 3, the complex is not precipitable by virus antibodies although it combines with them.

4. At a constant virus concentration the albumin/virus ratios in the complex increase with increasing concentration of albumin, the ratios decrease with increasing virus concentration when the albumin concentration is kept constant, the ratios increase with increasing total protein concentration when the proportion of the constituents remains constant, and with increasing temperature when all other conditions are constant.

5. Under similar conditions about 2.4 times as much albumin combines into a complex with heat denatured tobacco mosaic virus as with bushy stunt virus.

6. Heat denatured albumin molecules aggregate more rapidly with particles of heat denatured viruses than with one another.

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Nitrogenous Excretion in Chelonian Reptiles

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It seems to be well established that, during the course of evolution, the migration of animals from aquatic to terrestrial environments has been at least partially dependent upon their ability to detoxicate the ammonia arising from the α -amino nitrogen of the food proteins (Baldwin, 1948). Among vertebrates, detoxication is usually accomplished by the formation of either urea or uric acid. Ureotelism occurs in the Amphibia and Mammalia, and uricotelism in the Sauropsida (snakes, lizards and birds).

The nitrogenous excretion of the Chelonian reptiles (tortoises and turtles) has not hitherto been systematically studied, and such data as are available are often contradictory. Magnus & Muller (1835) found evidence for the presence of urea in the urine of *Testudo nigrita* (*nigra*), while Schiff (1825) and Marchand (1845) similarly demonstrated the presence of urea in the urine of *T. denticulata* (*tabulata*). Both of these species are land dwellers. Several workers have studied *T. graeca*, another terrestrial form, Clementi (1929) stating that 90% of the excreted nitrogen is in the form of urea, an observation which is substantially supported by the results of Drilhon & Marcoux (1942). Munzel (1938), on the other hand, found that uric acid and urea were both produced in this species, the former predominating. He also analyzed the urine of the semi-aquatic species, *Emys orbicularis* (*europaea*) and found that the main excretory product was urea, uric acid accounting for only a small proportion of the nitrogen excreted. In both the species studied Munzel (1938) was able to demonstrate the synthesis of urea by liver slices, and, in *Testudo graeca*, of uric acid also. *Chrysemys picta*, which is also semi-aquatic, was studied by Wiley & Lewis (1927) who found that the main

excretory product was urea, although ammonia and uric acid were also present in considerable quantities. Both Lewis (1918) and Khalil (1947) have worked on the sea turtle, *Chelonia mydas*, but obtained contradictory results. Lewis (1918) found that this species is essentially ureotelic, whereas Khalil (1947) states that ammonia is the main excretory product. Needham (1931), basing his argument on the work of Clementi (1929), Lewis (1918), and Wiley & Lewis (1927), concluded that Chelonians are essentially ureotelic. He believes that, because they are laid in a damp environment, the eggs can absorb water from the surrounding mud or sand and are therefore presumably permeable to urea also. In many species, however, the eggs are laid in very dry surroundings where a permeable egg would hardly be able to conserve or acquire enough water for development.

It therefore seemed worth while to investigate the nitrogenous excretory products of Chelonians from a variety of habitats in an attempt to clarify this somewhat obscure position. Species from a variety of aquatic, amphibious and terrestrial habitats were therefore studied to discover whether there might be some correlation between nitrogen metabolism and environmental conditions.

EXPERIMENTAL

Material. Dr E. Hindle, F.R.S., was kind enough to put at our disposal the large collection of Chelonians belonging to the Zoological Society of London.

For the collection of urine the smaller specimens were kept and fed in rat metabolism cages of the usual type for 1 or 2 days. The wire floor separated the urine from the faeces, and fragments of food were removed by filtration through a loose plug of glass wool. The urine was collected in bottles containing a small amount of 0.2N H₂SO₄. The analysis

of urine collected in this way was usually begun within 24 hr of excretion, but when longer waiting was necessary a few drops of toluene were also added, and the whole kept in the refrigerator

Larger specimens were kept in their usual surroundings, as it was found that the excitement caused by being handled was usually sufficient to induce urination. Analysis of this urine was begun within a few hr of its excretion and toluene added, if it had to be kept overnight.

The urine in all species was clear except in *Testudo graeca* which produced a cloudy urine, possibly because of the large amount of uric acid present.

Methods. Total N was estimated by the Kjeldahl method, digestion was continued for 16 hr with the catalyst of Chibnall, Rees & Williams (1943), and the NH_3 formed determined by distillation in the apparatus of Markham (1942). Free NH_3 was also estimated by this method, the distillate in both cases being collected in a 2% (w/v) boric acid solution containing indicator as described by Sobel, Hirschman & Besman (1945). Urea was determined by incubation with urease (prepared from jack bean meal by extraction with 0.3% (w/v) KH_2PO_4 , followed by distillation of the NH_3 produced). Uric acid was estimated colorimetrically, 2 ml each of the urea and cyanide solutions as used by Brown (1945) were added to the sample followed by 1 ml of the arsenophosphotungstic reagent of Benedict & Franke (1922) and the whole diluted to 50 ml. Readings were made after 50 min.

Amino acids were collectively determined by a slight modification of the method of Sobel *et al.* (1945), the NH_3 formed was distilled by Markham's (1942) method instead of being removed by aeration. Creatinine was estimated by the Folin picrate method, using the sodium picrate solution recommended by Borsook (1935) and hippuric acid by the colorimetric method of Denigès (1939). Before estimating either allantoin or guanine + xanthine it is necessary to uric remove acid, which gives a colour in both the colorimetric methods used. This was achieved by heating in a boiling water bath for 10 min with 0.2N nitric acid as

recommended by Hitchings (1941). Allantoin was then estimated by the colorimetric method of Young & Conway (1942), while guanine + xanthine were precipitated as silver salts by the method of Gulland, Jordan & Threlfall (1947), and estimated colorimetrically with the phenol reagent of Folin (1927) as used by Hitchings (1941).

RESULTS

Eight species of Chelonian from a variety of habitats were examined. The averages of the results for each species are tabulated in Table 1.

Kinosternon subrubrum, an omnivorous scavenger, and *Pelusios derbianus*, a carnivore, although belonging to different families, resemble each other in being almost wholly aquatic. Both species feed in water, and, indeed, it is said that *Kinosternon subrubrum* can be kept for years in a deep tank with no possible means of leaving the water and still remain in good condition (Ditmars, 1922). In both these species ammonia and urea are excreted in approximately equal quantities, each accounting for 20–25% of the total nitrogen excreted while the uric acid level is low at less than 5%.

Emys orbicularis is a semi aquatic species and differs from *Kinosternon subrubrum* and *Pelusios derbianus* in that it feeds on land, although it never leaves the marshy ground near the streams in which it spends much of its life. This species excretes only 15% of its waste nitrogen as ammonia, here urea is the predominant end product and accounts for 50% of the nitrogen excreted. Uric acid production again accounts for less than 5% of the total nitrogen.

Kinixys erosa and *K. youngi* are terrestrial species. *K. erosa* is restricted to damp habitats and frequently enters the water, but *K. youngi* is found

Table 1. Nitrogen partition in excreta of Chelonian reptiles

(Where more than one determination was carried out the non bracketed figures are mean values, whilst the figures in brackets show extreme values obtained.)

Species	No of determinations	Total N (mg/sample)	Nitrogen as percentage of total nitrogen							Percentage total N accounted for
			Ammonia	Urea	Uric acid	Amino acids	Allantoin	Guanine + xanthine	Creatinine	
<i>Kinosternon subrubrum</i>	1	7.0	24.0	22.9	0.7	10.0	1.1	1.0	Trace	59.7
<i>Pelusios derbianus</i>	1	9.5	18.5	24.4	4.5	20.6	1.0	3.8	Trace	72.8
<i>Emys orbicularis</i>	3	12.9	14.4 (10.6–18.8)	47.1 (26.4–69.0)	2.5 (1.4–4.0)	19.7 (15.8–21.9)	0.6 (0.3–0.9)	1.0 (0.5–1.2)	Trace	85.2
<i>Kinixys erosa</i>	2	16.0	6.1 (5.7–6.5)	61.0 (50.0–72.0)	4.2 (3.3–5.0)	13.7 (12.7–14.7)	0.4	0.4	Trace	84.8
<i>K. youngi</i>	5	13.3	6.0 (2.9–12.5)	44.0 (30.3–72.7)	5.5 (3.4–8.7)	15.2 (7.7–22.0)	0.8 (0.3–1.3)	2.1 (0.3–3.8)	Trace	73.6
<i>Testudo denticulata</i>	3	13.9	6.0 (3.9–8.7)	29.1 (26.8–31.2)	6.7 (4.9–7.7)	15.6 (9.4–22.2)	7.7 (6.7–8.6)	2.8 (1.7–5.0)	Trace	67.9
<i>T. graeca</i>	2	13.6	4.1 (4.0–4.1)	22.3 (15.3–29.3)	51.9 (48.7–55.0)	6.6 (4.5–8.6)	1.7 (1.4–1.9)	9.4 (8.7–10.1)	Trace	96.0
<i>T. elegans</i>	2	21.1	6.2 (5.9–6.5)	8.5 (7.8–9.1)	56.1 (54.3–57.8)	13.1 (10.6–15.5)	1.1 (1.0–1.2)	3.0 (0.9–5.1)	Trace	88.0

under much drier conditions. The nitrogenous excretion of the two species is, nevertheless, very similar. Both excrete 40–60 % of the total nitrogen as urea, ammonia is responsible for only 6 % of the excreted nitrogen and the uric acid level remains at 5 %.

Three species of the wholly terrestrial family Testudinidae were studied and here an interesting division was discovered. *Testudo denticulata*, which inhabits damp swampy ground in Brazil, excretes some 30 % of its total nitrogen as urea and only 7 % as uric acid, but *T. graeca* and *T. elegans*, both of which live under almost desert conditions, excrete uric acid as their main nitrogenous end-product. Although these species excrete 50–60 % of their total nitrogen as uric acid, they still produce considerable quantities of urea (10–20 %) in contrast to other uricotelic forms where urea synthesis is completely suppressed.

Apart from ammonia, urea and uric acid, amino-acids accounted for 10–20 % of the total nitrogen in all species. Allantoin forms 1–2 % of the total (except in *T. denticulata* where it accounts for 8 %), guanine + xanthine was consistently low at less than 4 % (except in *T. graeca* where it accounted for 9 % of the total), and creatinine was found in traces only. Hippuric acid, which is 10–25 % in Khalil's (1947) analysis of the urine of *Chelonia mydas*, was not detectable in any of the species studied.

In all, 60–95 % of the total nitrogen excreted has been accounted for in each species.

DISCUSSION

The Chelonian reptiles seem to be biochemically separable into three groups. The first group contains the aquatic and semi-aquatic species, and these excrete both ammonia and urea in approximately equal quantity. In the second group, consisting of those terrestrial species which are restricted to damp, marshy ground, there is little excretion of ammonia but some 50–60 % of the total nitrogen is excreted as urea. The third group includes the terrestrial species which live in very dry habitats and which excrete some 60 % of their waste nitrogen in the form of uric acid. Although this group is essentially uricotelic, the ureotelic habit of *Chelonia* in general has not been completely suppressed, for about 10–20 % of the total nitrogen is still excreted as urea. The family Testudinidae seems to be the most interesting from this point of view, some of its species being typically ureotelic while others excrete urea and uric acid together, the latter predominating.

According to Needham (1931), the change from ureotelism to uricotelism is determined by the conditions under which embryonic development takes place, uricotelism being associated with development within a 'cleidoic' egg. The eggs of the ureotelic

species *Thalassochelys corticata* (Karashima, 1929), *Emys orbicularis* (*europaea*, see Hochstetter, 1906), and *Caretta* (Hildebrand & Hatzel 1927), all require a damp environment for successful development. Karashima's (1929) data show that the eggs of *Thalassochelys corticata* absorb water to the extent of 42 % of their original water content from the environment during development, and Nakamura (1929), also working on this species, showed that the total nitrogen of the egg decreased by 17 % before hatching. These eggs are therefore permeable to water and probably to urea also, so that the urea formed by the embryo can diffuse out into the environment. In addition, certain other ureotelic species such as *Chrysemys picta picta* (Cunningham, 1923), *C. picta bellii* (Stromsen, 1923), *Emys orbicularis* (Rollinat, 1932), and several others moisten the ground with fluid from the bladder or some closely associated gland before laying their eggs. The reason for this may be to produce an artificially damp environment for the eggs, but it has been suggested (Stromsen, 1923), that it may be only a method of softening the ground to facilitate the digging of the nest.

Young's (unpublished) work on the egg of *Testudo graeca*, however, shows conclusively that in this species the shell is even more impermeable than that of the domestic fowl. The eggs, which develop under very dry conditions, are presumably supplied with sufficient water to last through their embryonic life. Being impermeable to water, they are almost certainly impermeable to urea also. The urea produced by a ureotelic embryo would therefore accumulate within the egg, and perhaps might reach a toxic level. It is in those species like *T. graeca*, which live and lay their eggs in almost desert habitats, that uricotelism has largely replaced ureotelism. It seems probable, therefore, that this is an adaptation to embryonic life within a 'cleidoic' egg, but further data on the permeability and conditions of hatching of the eggs of other species are required to confirm this view. If, as appears probable, it is found that the development of uricotelism takes place side by side with the development of a 'cleidoic' egg within this group, this would be further evidence in support of Needham's (1931) belief that the two factors are interdependent.

This biochemical evidence supports the usual theory of Chelonian evolution based on morphological and palaeontological grounds. The tortoises and turtles are believed to have evolved from an early amphibious stock which must presumably have been ureotelic. Some of these early forms returned to an aquatic environment where they again began to excrete some ammonia, while retaining partially their ureotelic nature, thus giving evidence of their amphibious ancestry. Others remained in damp terrestrial habitats and are still typically ureotelic.

A third group migrated to drier terrestrial habitats, and here, apparently because of the conditions of embryonic development, they became largely uricotelic. In the Sauropsida, which are all uricotelic, very little urea indeed is produced, and this is not of synthetic origin, being produced from exogenous urea found in the food and from the amidine group of arginine (Clementi, 1932). The fact that these Chelonian species, while being mainly uricotelic, still excrete some 10–20 % of their total nitrogen as urea, suggests that the stable uricotelic state found in the Sauropsida has not yet been achieved. It may be that their biochemical evolution towards uricotelism is still in progress.

SUMMARY

1 The nitrogenous excretion of eight species of Chelonian reptiles was studied.

2 The analyses showed that urea accounted for 10–20 %, or more, of the total nitrogen excreted by all the species examined.

3 While the amphibious species were found to be typically ureotelic, the aquatic forms excreted approximately equal quantities of ammonia and urea, and the xerophilous, terrestrial species, excreted uric acid as their main nitrogenous end product.

4 The significance of these observations is discussed in relation to the environmental conditions which prevail in adult and embryonic life.

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The Concentration and Distribution of Haemoglobin in the Root Nodules of Leguminous Plants

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All legume nodules which are actively fixing nitrogen contain a red pigment. This pigment escaped attention until Pietz (1938) suggested that it was identical with dihydroxyphenylalanine (Dopa), the red intermediate in the enzymic oxidation of tyrosine. Kubo (1939), however, prepared a crude extract of the pigment and from its spectroscopic behaviour identified it as a haemoprotein possessing properties very similar to those of haemoglobin. Burriss & Haas (1944) stated that the pigment was not a haemoglobin but a haemoprotein oxidation-reduction catalyst. However, Keilin & Wang (1945), working on an extract about 50 % pure, were able to confirm the haemoglobin-like nature of the pigment by showing that it was capable of completely reversible oxygenation and deoxygenation, they determined the absorption spectra of the oxygenated and reduced haemoglobin and those of some of its derivatives. Keilin & Wang suggested that the

the case of the property of nitrogen fixation. Neither the host plant nor the root-nodule bacteria cultivated alone will fix nitrogen. Those micro-organisms which fix nitrogen when free living (*Nostoc*, *Azotobacter* and *Clostridium pasteurianum*) do not possess haemoglobin. In a preliminary examination of the root nodules of alder, which are also believed to fix nitrogen, the author was unable to detect the pigment. There is, however, indirect evidence that the haemoglobin in the nodule is concerned in the process of symbiotic nitrogen fixation. This may be summarized as follows: (1) Haemoglobin is present in nodules of all the leguminous plants which actively fix nitrogen. (2) Haemoglobin is absent from nodules produced by certain ineffective strains of *Rhizobium* and which fix very little nitrogen. (3) Symbiotic nitrogen fixation is inhibited by a concentration of carbon monoxide much lower than that which is required to inhibit fixation by *Nostoc* or *Azotobacter*.

Table 1 *The positions of the maxima of the absorption bands of nodule haemoglobin and some derivatives*

(Values given by Keilin & Wang, 1945)

	Maxima (in mμ)		
	α		β
Hb		557	
HbO ₂	574		540
HbCO	564		538

failure of Burriss & Haas to recognize the pigment as a haemoglobin was due to the fact that in their preparations the pigment had become largely oxidized to methaemoglobin. Table 1 shows the positions of the maxima of the absorption bands of reduced, oxidized and carboxy haemoglobin (Hb, HbO₂ and HbCO). Working at 15°, Keilin & Wang found the pO₂ giving 50 % dissociation of HbO₂ to be less than 0.1 mm Hg, and the relative affinity for CO and O₂ ($K = \frac{[\text{HbCO}][\text{pO}_2]}{[\text{HbO}_2][\text{pCO}]}$) to be 37.

A remarkable fact about nodule haemoglobin is that neither the root nodule bacteria nor the host plant is able to produce it when grown separately. It is only found in the nodule which is produced after infection of the host legume with an appropriate strain of *Rhizobium*. A similar relationship holds in

the case of the property of nitrogen fixation. Neither the host plant nor the root-nodule bacteria cultivated alone will fix nitrogen. Those micro-organisms which fix nitrogen when free living (*Nostoc*, *Azotobacter* and *Clostridium pasteurianum*) do not possess haemoglobin. In a preliminary examination of the root nodules of alder, which are also believed to fix nitrogen, the author was unable to detect the pigment. There is, however, indirect evidence that the haemoglobin in the nodule is concerned in the process of symbiotic nitrogen fixation. This may be summarized as follows: (1) Haemoglobin is present in nodules of all the leguminous plants which actively fix nitrogen. (2) Haemoglobin is absent from nodules produced by certain ineffective strains of *Rhizobium* and which fix very little nitrogen. (3) Symbiotic nitrogen fixation is inhibited by a concentration of carbon monoxide much lower than that which is required to inhibit fixation by *Nostoc* or *Azotobacter*.

From these facts alone there can be little doubt that haemoglobin plays a role in nodular nitrogen fixation and in this paper further evidence based on the distribution of the pigment in the nodule will be presented. The mechanism into which haemoglobin enters is as yet unknown. Virtanen and his co-workers (Virtanen & Laine, 1946) claimed that, in addition to haemoglobin, methaemoglobin was present in nodules, and put forward a theory of nitrogen fixation in which haemoglobin functioned as an oxidation-reduction catalyst. However, on repeating Virtanen's experiments, Keilin & Smith (1947) were unable to find any evidence for the presence of methaemoglobin in nodules and from this and other considerations rejected Virtanen's theory. The suggestion that haemoglobin may, by virtue of its oxygen carrying property, take part in the oxygen uptake of nodules will be discussed in the following paper. In the present paper the distribution and concentration of the pigment in various types of nodule will be described.

METHODS

Cultivation of the root nodule bacteria. The *Rhizobium* strains were grown at 28 or 30° in pure culture on an agar medium (K₂HPO₄, 0.5 g, MgSO₄ 7H₂O, 0.2 g, NaCl, 0.2 g, CaCl₂, 0.2 g, FeCl₃, 0.001 g, Difco yeast, 5 g, agar, 15 g, distilled

water to 1 l). By using this medium in which a yeast extract preparation is the sole source of carbon and nitrogen, one avoids the copious gum production always encountered with media containing mannitol. Nodules on soya beans were produced by the effective *Rhizobium* strain 505 and the ineffective strain 507. These two strains are identical with those used by the Wisconsin group of workers (Wilson, 1940). In some experiments the strain 2193 (National Collection of Type Cultures), which infects peas, was used.

Growth of the plants Legumes were grown in pots containing sterilized soil to which had been added a suspension of cells of the appropriate strain of *Rhizobium*. Sufficient precaution against contaminant infections was obtained by placing plants in separate parts of the glasshouse (see p. 589).

Cytological methods Nodules were fixed in Bouin's fixative or in formalin acetic alcohol. These were sectioned, and stained either with iron haematoxylin and orange G as counterstain, or with carbol fuchsin. The sections stained with iron haematoxylin were more satisfactory.

The estimation of haemoglobin in nodules

Direct observation of haemoglobin Haemoglobin may easily be demonstrated in legume nodules using the microspectroscope. Whole nodules are too opaque for this observation, but on slicing or crushing the nodule the twin absorption bands of oxyhaemoglobin with maxima at 574 and 540 m μ are seen. On standing these are slowly replaced by the single band of deoxygenated haemoglobin at 557 m μ . This is due to respiratory activity of the nodule tissue.

General method of estimation For this purpose use was made of the microspectroscope and wedge trough as described by Keilin (1933) and by Keilin & Wang (1946). In this instrument the spectra produced by light from two different sources are compared. Light from one source passes through a double wedge trough, one half of which contains a standard solution, the other water. The optical depth of the standard solution through which light is passing is varied by movement of the wedge trough and is proportional to the distance through which this is moved. Light from the other source passes through a cylindrical vessel containing the solution of unknown density. In matching, two adjustments are made simultaneously: the intensities of the absorption bands in the two spectra are matched by movement of the wedge trough, and the intensities of the spectral backgrounds adjacent to the bands are matched by varying the strengths of the two light sources. If the matching is carried out in this manner it can be shown that opaqueness of the unknown solution does not affect the result, and measurements may in fact be made of concentrations of pigments in slices of tissue. In this way the haemoglobin was estimated as pyridine haemochromogen, both in aqueous extracts and directly in nodule slices.

Determination of nodule haemoglobin in aqueous extracts Nodules freshly removed from the plant were washed, and the surface moisture was removed by filter paper, the nodules were then weighed and the total volume of the nodule sample measured by placing in water and measuring the volume of water displaced. The nodules were then ground in water and the extract centrifuged to remove cellular debris, which was again extracted with water. The second extract was found to contain less than 2% of the total haemoglobin and was consequently discarded. The first extract was made up to a volume of 5 ml and a measured volume (v) of the solution placed in a cylindrical upright tube of cross

sectional area (a) on the stage of the microspectroscope. The haemoglobin was then converted to pyridine haemochromogen by the addition of a little NaOH (to denature the proteins), pyridine and $\text{Na}_2\text{S}_2\text{O}_4$. The comparison was made against a standard pyridine haemochromogen solution (of concentration C) contained in the wedge trough. The concentration of the unknown solution (c) is given by the relation $c = H C/h$, where H is the optical depth of the standard solution (this is obtained from the distance through which the wedge trough has been moved and from constants dependent on its dimensions), and h is the optical depth of the unknown solution ($=v/a$).

It will be shown that the haemoglobin is limited to the bacteria containing cells in the central part of the nodule, so, in order to calculate the approximate concentration of haemoglobin in these cells, correction must be made for the volume of the outer cortical parenchyma of the nodule which contains no haemoglobin. This volume was estimated from measurements of the relative areas of internal nodular tissue and cortical parenchyma in thin slices of nodules, assuming these to be spherical. These measurements were made using a microscope with a micrometer eyepiece.

Spectroscopic examination of the aqueous extract and the cellular debris showed that practically all the haemoglobin passed into the first extract. Only haemoglobin could be identified in this extract. The solid cellular debris showed only the absorption bands of components of the cytochrome system.

As the central nodular tissue consists both of cells containing bacteria and haemoglobin, and small interstitial cells devoid of both, the value obtained for the concentration of haemoglobin in the former cells is slightly less than the true value. This error, however, should not be greater than about 10%.

Determination of haemoglobin directly in nodule slices In this method all the haematin in a small slice of nodule was converted into pyridine haemochromogen and this determined directly by comparison with a standard haemochromogen solution. For this purpose each nodule was cut into slices 1–2 mm thick. A slice was placed on a glass slide in a few drops of pyridine and reducer ($\text{Na}_2\text{S}_2\text{O}_4$). A piece of plane glass was placed on top of the slices and supported at the corners by small pieces of plasticine. The whole was then compressed slightly so that all surfaces of the slices were in contact with the glass, and the two pieces of glass were adjusted to make their surfaces accurately parallel. This system was then placed on the stage of the microspectroscope, to which a low power objective had been added, so that the central part of the nodule was under the objective. The slices were left in contact with pyridine and reducer until the maximum amount of haemochromogen had developed (usually after 20 min). The mean of several readings over different parts of the nodule slice was taken. Then, as before, $c = H C/h$, where h is the thickness of the nodule slice. This was obtained by measuring with a micrometer the distance apart of the two pieces of glass with and without the slice in position.

Accuracy in this method requires (1) careful measurement of the thickness of the nodule slice, (2) evenness in the thickness of the slice, (3) that the light passing through the slice should be parallel.

The concentration measured in this way gives an upper limit to the amount of haemoglobin in the nodule, as haematin from the cytochromes and other haemoproteins is included in the estimation.

It might be thought that, since the haemoglobin is not distributed uniformly throughout the tissue, but is present in a number of small spherical cells, a false estimate of concentration would be obtained. However, it was found that the concentration of haemoglobin, when measured in a suspension of horse red blood cells under conditions when these were ellipsoidal in shape, was identical with that found after lysing the cells. The behaviour of this model suggests that the localization of the haemoglobin does not affect the estimation described above.

RESULTS

Localization of haemoglobin within the nodule

After the invasion of the root by the bacterial infection thread a meristem is set up in the root cortex. This meristem produces two kinds of cells, cells which later enlarge and are packed with bacteria, and smaller interstitial parenchymatous cells devoid of bacteria. The mature nodule then consists of an outer bacteria-free cortex and an inner tissue made up of the large bacteria-containing cells together with the interstitial cells (Fig. 1). In certain types of nodule these bacteria-containing cells undergo a rapid disorganization which is easily seen on cytological examination. Thornton (1939) has shown from measurements on such nodules that the rate of nitrogen fixation is proportional to the volume of bacteria-containing cells which have not undergone disorganization. It is reasonable to suppose that, since the nitrogen gas is actually taken up by the nodule, in these cells lies the seat of nitrogen fixation. It is interesting therefore to know whether the haemoglobin is present in these cells.

In order to answer this question soya nodules (*Rhizobium*, strain 505) were examined. From observation of the distribution of the red colour in cut nodules, and by careful examination of thin slices of nodules under the microspectroscope, it was apparent that there was no haemoglobin in the cortex. Use was then made of the peroxidase action of haemoglobin to determine its localization within the nodular tissue proper. If an ethanolic solution of benzidine together with hydrogen peroxide is added to an acid haemoglobin solution, the benzidine is oxidized to a substance with a quinonoid structure which polymerizes giving a deep blue compound. While some very strong oxidizing agents bring about a similar reaction, no such substances are found in living cells, and the formation of a blue colour under these conditions indicates the presence of haemoglobin. Under acid conditions peroxidase itself will not bring about this reaction and its presence in plant cells will not interfere with the detection of haemoglobin by this method. This reaction was tested on thin sections of soya nodules cut with a hand razor. In these sections the cytoplasm and often the nuclei of the large bacteria-containing cells were stained blue, while all other cells were unstained (Fig. 2).

Haemoglobin within the nodule is thus confined to those cells which contain bacteria and which are probably specifically concerned with nitrogen fixation.

If nodules are ground up in water, and the bacteria and insoluble debris centrifuged down, spectroscopic examination shows all the haemoglobin to be in solution. The pigment is not present in the bacteria, but diffused throughout the vacuole and cytoplasm.

The appearance of haemoglobin in young nodules

In the early development of the nodule there is a period when the meristem has begun to cut off the cells which will make up the bacteria-containing tissue, but during which the differentiation of these cells is still incomplete. At this time the future bacteria-containing cells are small, round and little distinguished from the interstitial cells. Observations were made to find out at what stage in nodular development detectable quantities of haemoglobin appear.

Soya nodules (*Rhizobium*, strain 505) were sampled 4 weeks after plant inoculation. Nodules of various sizes were sliced, and the slices examined for haemoglobin by direct observation of the presence or absence of a red colour (the nodules were too small to be examined under the microspectroscope). They were divided into two groups, those which were red on slicing and those which were white. Nodules from each group were fixed, sectioned and stained with iron haematoxylin.

All those nodules which possessed a detectable amount of haemoglobin also showed a well developed bacteria-containing tissue, the large cells of which contained many bacteria and were easily distinguished from the interstitial cells. In nodules in which no haemoglobin could be observed, differentiation of the bacteria-containing region had not yet begun or was just commencing. The cells in such nodules contained few bacteria (Figs. 3 and 4).

Using this crude method of detecting haemoglobin it was possible to show that the time of appearance of a large amount of haemoglobin in the nodule more or less coincides with the differentiation of the bacteria-containing region and never precedes it.

The amount of haemoglobin in effective nodules

When it had been shown that the haemoglobin is restricted to the bacteria-containing cells, the concentration of haemoglobin within these cells was measured.

Haemoglobin in nodules of bean (Phaseolus vulgaris dwarf variety) and soya

Using the microspectroscope and wedge trough the amounts of haemoglobin in effective nodules from two species of legumes were measured. The

bean plants were harvested in April 1947 when flowering and bearing fairly large nodules. The soya plants (inoculated with *Rhizobium*, strain 505) were harvested in June 1947, just before flowering, and the nodules were placed according to size into two groups. Table 2 gives the data and the results of

Haemoglobin and haematin compounds in ineffective nodules

Different strains of *Rhizobium* may produce nodules on the same host plant species with greatly varying nitrogen-fixing activity, some strains giving

Table 2 *Haemoglobin content of nodules as determined in aqueous extracts*

Plant	Fresh wt of sample (g)	Mean fresh wt of single nodule (mg)	Volume of nodule sample (ml)	Haematin in extract (mg)	Mean value of the ratio Volume of bacteria containing cells Total volume	Concentration Hb in bacteria containing cells (as haematin) (10^{-4} M)	Hb _{17,000} * Fresh wt nodule (mg/g)	Mean* Hb _{17,000} nodule (mg)
Bean	1.7	16.8	1.7	0.072	0.59	1.09	1.09	0.0183
Soya (medium sized nodules)	0.866	8.7	0.85	0.088	0.38†	4.2	2.68	0.0233
Soya (small nodules)	0.404	4.9	0.40	0.050	0.38†	5.0	3.25	0.0157

* The amount of haemoglobin is calculated as haemoglobin units of molecular weight 17,000

† Independent of nodule size. Standard error in each case is $\pm 9.7\%$ of mean value.

Table 3 *Haemoglobin (including haematin from cytochromes) as determined in bean nodule slices*

Nodule no	Concentration of Hb as haematin in bacteria containing cells ($\times 10^{-4}$ M)
1	2.5
2	1.72
3	3.76
4	1.28
5	2.17
6	2.59

Mean 2.34 (S.E. $\pm 15\%$)

measurements carried out in aqueous extracts, and Table 3 records measurements made on individual nodule slices from a single sample of bean nodules.

While the concentration of haemoglobin in different nodules is subject to considerable variation the values are all of the same order of magnitude. Those obtained from measurements of total haematin in nodule slices give an upper limit to the amount of haemoglobin in the nodule, and are consequently greater than values from measurements in aqueous extracts. The bean nodules were grown in April 1947, those of soya in June 1947. Because of this difference in time of measurement, it is not certain whether the greater amount of haemoglobin in the soya nodules should be attributed to differences in cultural conditions or to a difference between the two genera. Within the range examined there appears to be little variation in haemoglobin content of nodules of different sizes. It is probable that the haemoglobin content of the bacteria-containing cells remains constant after their differentiation until the onset of their disintegration, but when the latter occurs the haemoglobin eventually disappears and may be replaced by a green pigment (Virtanen, Laune & Linkola, 1945; Virtanen & Laune, 1946).

rise to nodules which fix practically no nitrogen. Such strains are called ineffective strains and the nodules they produce will be referred to as ineffective nodules. Ineffective nodules are usually smaller than effective nodules and are distributed more evenly over the entire root system. Examined cytologically the central tissue is seen to be quite different from that of normal nodules, while both cortex and interstitial parenchymatous cells are entire, the large bacteria-containing cells appear in a state of breakdown. Usually the nuclei have disappeared, and the cytoplasm is rounded off into large masses containing many bacteria, mainly small cocci. The ratio of uninfected cells to infected cells in the central tissue of such nodules is characteristically greater (Fig. 5).

Thornton (1939) finds that in the early stages of development of the most ineffective nodules there is some organized bacteria-containing tissue, but that the difference between these and effective nodules lies in the stability of this central tissue. Once disintegrated it ceases to fix nitrogen. This is borne out by Thornton's experiments, in which, by cytological studies and Kjeldahl analyses on nodules and plants grown under sterile conditions (ensuring purity of the infecting *Rhizobium* strain), he showed that the amount of nitrogen fixed by nodules of varying effectiveness is proportional to the amount of organized central tissue and its duration. The nodules produced by strains of *Rhizobium* isolated by Virtanen (1947), which he claims fix no nitrogen, are probably distinguished by the extreme rapidity with which the cells of the central nodular tissue undergo disintegration. Virtanen's suggestion that the low nitrogen-fixing power of ineffective nodules is due to the absence of the irregularly shaped bacterial forms, usually referred to in the literature as bacteroids, has been criticized by Thornton who points out that

bacteroids may be observed in young ineffective pea and clover nodules, and furthermore that bacteroids are absent in all nodules formed on soya plants, whether effective or ineffective. There is no good reason to connect bacteroids with the process of nitrogen fixation. However, the low rate of nitrogen fixation by ineffective nodules is always associated with the breakdown of the nodular tissue.

Virtanen (1945) has shown that haemoglobin cannot be detected in ineffective nodules. Instead a green pigment is often found which, as Virtanen *et al* (1945) and Virtanen & Laine (1946) have shown, may be similar to certain of the bile pigments formed during haemoglobin breakdown in animals. This green pigment is of little interest in the study of the nitrogen fixation process since it is invariably formed

The haemoglobin was extracted in water from samples (1–2 g) of both types of nodule and estimated as pyridine haemochromogen as previously described. Only bands of haemoglobin could be detected when the aqueous extract from effective nodules was examined under the microspectroscope. No spectroscopically distinguishable components were visible in the aqueous extract from ineffective nodules although this extract contained a green pigment. As will be seen, however, haematin compounds were present in small amounts in this extract. When the solid debris remaining after centrifuging was examined under the microspectroscope in the presence of a little reducer ($\text{Na}_2\text{S}_2\text{O}_4$), only bands of the cytochromes could be seen in each case, but these bands were much stronger in the debris from effective

Table 4 *Haematin content of effective and ineffective nodules*

	Effective nodules (<i>Rhizobium</i> , strain 505)		Ineffective nodules (<i>Rhizobium</i> , strain 507)	
	(mg haematin/g fresh weight of nodules)	Percentage of total	(mg haematin/g fresh weight of nodules)	Percentage of total
Haematin in aqueous extract	0.071	92.2	0.0068	78.2
Haematin in residue	0.006	7.8	0.0019	21.8
Total haematin	0.077	—	0.0087	—

The ratio (total haematin in nodules of strain 507/total haematin in nodules of strain 505) was 0.113

after the nodule has ceased to fix nitrogen. Since it appears in considerable quantities in nodules which have never contained detectable amounts of haemoglobin, it is difficult to understand how it originates from the breakdown of haemoglobin, unless this is being produced and broken down almost simultaneously. Alternatively, haemoglobin may not be produced, but instead quantities of haematin which are transformed to the green pigment.

To find out in what way ineffective nodules are distinguished from effective nodules some measurements of the total amounts of haematin in effective and ineffective nodules and of haematin production by both types of strain of the nodule bacteria were made.

Haemoglobin and total haematin in effective and ineffective soya nodules

Three groups each of forty soya plants were grown in open pots in sterilized soil, each group in a separate part of the greenhouse. One group was inoculated with *Rhizobium* strain 505 (effective), the second group with strain 507 (ineffective), while the third (control) group of plants was left uninoculated. None of the control plants produced nodules, consequently it may be assumed there were no contaminant infections. The plants were harvested 4 weeks after inoculation (June 1947).

The amounts of haematin in these solid residues were also measured. Table 4 lists all these results. The haematin in the solid residue in both cases comes from the cytochromes and small amounts of other haemoproteins. The haematin in the aqueous extract of 505 nodules is derived almost entirely from haemoglobin. That found in the 507 aqueous extract may originate in any of three ways: (a) certain of the cytochromes may pass more readily into solution because of the tissue breakdown in these nodules, (b) free haematin or unidentified haematin compounds may be present, or (c) there may be in 507 nodules a quantity of haemoglobin too small to be detected by direct observation.

*The cytology of ineffective soya nodules (*Rhizobium*, strain 507)*

In sections of ineffective nodules the general disintegration of the whole nodule tissue was very marked, more especially affecting the large bacteria-containing cells. The nuclei of these had disappeared and the cytoplasm was breaking up into fragments containing large numbers of bacteria. The cytoplasm of the interstitial cells was also undergoing disorganization and groups of bacteria were associated with the cell walls which were breaking down. The relative number of interstitial cells was much greater in this type of nodule. Younger ineffective nodules

showed only the initiation of these processes, but haemoglobin could not be detected in these, however young

In the effective nodules produced by *Rhizobium*, strain 505, no such disorganization could be seen even in large nodules (Figs 1 and 5)

Haematin and cytochrome in pure cultures of Rhizobium

The great difference in total haematin contained in effective and ineffective nodules suggested the possibility that *Rhizobium* strains might differ in their capacity to produce haematin

In one experiment pure cultures of the *Rhizobium*, strains 505 and 507, were grown at 28° on an agar medium containing inorganic salts and yeast extract. After 4 days (the soya organism belongs to the slow growing group of Rhizobia) the cells were harvested and washed in 0.1 M-phosphate buffer, pH 7.3

The intensities of the reduced cytochrome bands appeared about the same in both suspensions. Examination of these bands showed in each case the presence of cytochromes *a*, *b* and *c* with the *a* bands of components *b* and *c* extremely close together. On samples of the suspension the total haematin was determined as pyridine haemochromogen, and the nitrogen content by the micro Kjeldahl method using the Markham steam distillation apparatus. These results are given in Table 5

Table 5 *Haematin content of pure cultures of effective and ineffective strains of Rhizobium*

Strain no	Haematin content of suspensions (mg/100 ml)	Nitrogen content of suspensions (mg/ml)	Haematin (mg/mg N)
505	1.3	8.6	1.5×10^{-3}
507	0.86	8.6	1.0×10^{-3}

The amounts of haematin produced by these two strains are of the same order. The difference is small and does not account for the great difference in haematin content of the two types of nodule

DISCUSSION

In nodules produced by ineffective strains of *Rhizobium*, not only is the absence of haemoglobin paralleled by a correspondingly lower amount of extractable haematin as compared with that of effective nodules, but the amount of non extractable haematin compounds is also lower. This latter difference exists when the haematin content is calculated on a fresh weight or a dry weight basis (the ratio fresh weight/dry weight being the same

in each type of nodule). There is also less cytochrome in ineffective nodules. This may probably be accounted for by the smaller number of bacteria in such nodules.

When grown in pure culture, effective and ineffective strains of the root nodule bacteria do not differ greatly in the amounts of haematin they produce. The absence of haemoglobin and the failure to fix nitrogen are doubtless directly connected with the breakdown of the bacterial tissue, as these three phenomena occur together in nodules produced under other circumstances. Such types of nodule are: (1) Nodules formed by effective strains of *Rhizobium* on certain varieties of host plant (Nutman, 1946). These nodules have a white-green colour and thus can possess little haemoglobin (Nutman, private communication). (2) Nodules formed on boron deficient plants (Brenchley & Thornton, 1925). It is not known whether these nodules contain haemoglobin. (3) Effective nodules borne on plants nearing the end of their life cycle. (4) Nodules on plants which have been kept in the dark for a period of at least 11 days (Keilin & Smith, 1947).

Under the last three conditions the factor causing the breakdown of the nodular tissue is apparently shortage of carbohydrate. Bond (private communication) notes that nodules formed on plants grown in the dark but supplied with glucose are red in colour (in contrast to nodules formed under these conditions without an external supply of carbohydrate).

Taking into account the structure and properties of all these different types of nodule, it may be concluded that the production of haemoglobin and the ability to fix nitrogen are properties not resulting from the mere haphazard association of bacteria and host tissue in the nodule, but are dependent on a very precise relationship between the bacteria and the large cells in the nodular tissue in which they are found. The presence of haemoglobin, and the ability to form with the rest of the plant a nitrogen fixing system, are characteristics which do not arise until these cells reach a certain size, and both disappear when sooner or later in their development (depending on the conditions listed above) these cells undergo breakdown.

SUMMARY

1 Haemoglobin in legume nodules is contained only in the large bacteria-containing cells and does not appear in detectable amounts until these cells have been differentiated.

2 The concentration of haemoglobin in the bacteria-containing cells of nodules of different legume species varied between 1 and 5×10^{-4} M. After cell differentiation the concentration did not appear to vary with size of nodule.

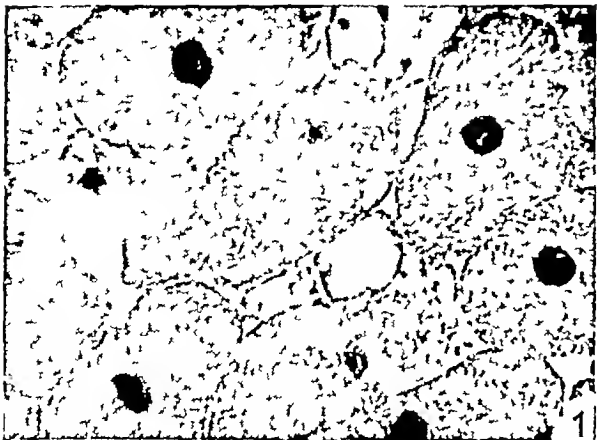


Fig 1



Fig 2

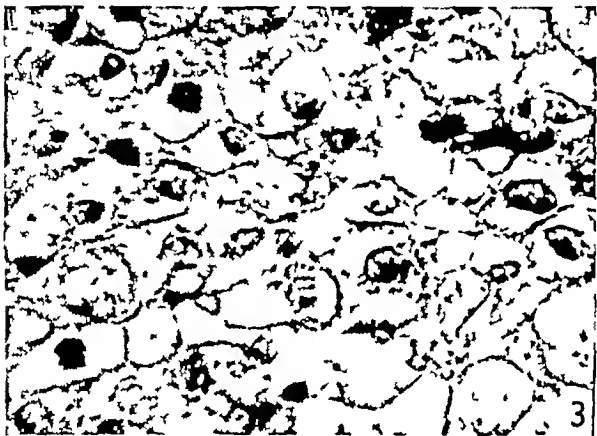


Fig 3

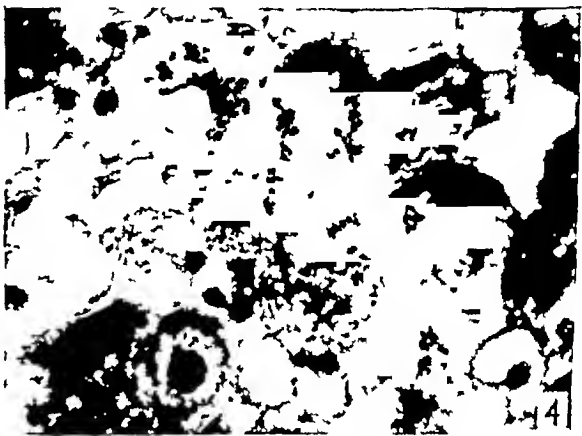


Fig 4



Fig 5

J D SMITH—THE CONCENTRATION AND DISTRIBUTION OF HAFMOGLOBIN
IN THE ROOT NODULES OF LEGUMINOUS PLANTS

3 As reported by Virtanen (1945), haemoglobin could not be detected in nodules produced by ineffective strains of *Rhizobium*. The amount of haematin in such nodules, estimated as pyridine haemochromogen, was much less than that in effective nodules.

4 Effective and ineffective strains of *Rhizobium*

grown in pure culture differ little in the ratios of haematin/cell nitrogen.

The author wishes to thank Prof D Keilin, F R S, for his advice and encouragement, Dr K Smith, F R S, for permission to carry out the work, and Dr H G Thornton, F R S, who supplied cultures of the *Rhizobium*, strains 505 and 507.

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EXPLANATION OF PLATE 6

Fig 1 Section through an effective soya nodule (*Rhizobium*, strain 505) showing the bacteria containing tissue (Iron haematoxylin and orange G) ($\times 800$)

Fig 2 Part of a section of a bean nodule treated with benzidine and hydrogen peroxide in acid solution ($\times 100$)

Fig 3 The central tissue of a soya nodule before the develop-

ment of detectable amounts of haemoglobin (Iron haematoxylin) ($\times 800$)

Fig 4 The central tissue of a soya nodule after the appearance of haemoglobin, showing well developed bacteria containing cells (Iron haematoxylin) ($\times 800$)

Fig 5 Section through an ineffective soya nodule (*Rhizobium*, strain 507) showing the bacteria containing tissue (Iron haematoxylin and orange G) ($\times 800$)

Haemoglobin and the Oxygen Uptake of Leguminous Root Nodules

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The relation between the partial pressure of oxygen and the functioning of the leguminous root nodule is twofold, as oxygen affects both nodular development and the rate of nitrogen fixation by the mature nodule. Nodules formed in the almost complete absence of oxygen are small, white, and contain no haemoglobin (Virtanen, 1947). They are also deficient in vascular strands, but do not undergo the cellular disintegration typical of ineffective nodules. Thornton (1930) has shown that such nodules fix little nitrogen. The effect of depriving normal mature nodules of their oxygen supply has been studied by Golding (1903) and by Virtanen & von Hausen (1935, 1936), who found that uptake of gaseous nitrogen in nutrient solution cultures ceased in the absence of an oxygen supply to the nodulated roots, while uptake of combined nitrogen was independent of root

aeration. Wilson & Fred (see Wilson, 1940) have produced quantitative results demonstrating the effect of growing entire clover plants in partial pressures of oxygen (pO_2) varying between 0.012 and 0.6 atm. They found that reduction of the pO_2 down to 0.012 atm was accompanied by a proportional decrease in the uptake of gaseous and combined nitrogen. Their experiments, however, are not directly comparable with those of Golding (1903) and Virtanen & von Hausen (1935, 1936), in which the pO_2 would probably be much lower than 0.012 atm and the green parts of the plants were in an atmosphere containing 20% oxygen.

Thus, apart from its effect on the development of the nodule, oxygen is concerned in the process of nitrogen fixation. It may merely be involved indirectly through the release of energy by the oxida-

tion of carbohydrates, or it may in addition enter directly into the fixation mechanism

A fair amount of information is available concerning the respiration of nodules. Two important experimental results must be kept in mind when considering this work (Wilson, 1940), (1) Bacteria from crushed nodules, separated from the tissue debris, behave, so far as their respiration is concerned, in a manner identical with those from pure cultures with the exception that the former, in the case of the faster-growing group of *Rhizobia*, attain their maximum rate of oxygen uptake at a lower pO_2 than do bacteria grown in pure culture (2) *Rhizobium* in pure culture will grow well at a pO_2 of less than 0.01 atm

The relative rates of respiration of nodules and roots appear to vary within fairly wide limits. All Q_{O_2} values in this paper are in $\mu\text{l}/\text{mg}$ dry wt/hr. Values for the ratio Q_{O_2} nodules/ Q_{O_2} roots between 0.5 and 3.1 have been reported by different workers (Allison, Ludwig, Hoover & Minor, 1940; Asprey & Bond, 1941; Wilson, 1940). Allison *et al.* found that sliced or crushed nodules had a much higher Q_{O_2} than whole nodules and that the respiratory quotient of nodules respiring in air was well in excess of 1, the value of the latter increasing with the size of nodule. Such results show that the pO_2 within the nodule must be very low and that oxygen uptake must be determined largely by the rate of diffusion of the gas through the nodule tissue. This is not a surprising fact considering that most nodules are large spheres, several mm in diameter, containing cells packed with bacteria.

In view of this, the possibility that the nodule haemoglobin may have some effect on oxygen uptake by the nodular tissue at these low partial pressures of oxygen must be considered. Three ways in which haemoglobin could have such an effect suggest themselves.

(1) Haemoglobin might act as a store of oxygen. However, a simple calculation shows that haemoglobin could not act as such a store over any length of time. From the data presented in the preceding paper (Smith, 1949) it can be calculated that the haemoglobin in 1 g (fresh wt) of the bacteria-containing cells of soya nodules, if completely oxygenated, would contain 11.2 μl of oxygen. The mean Q_{O_2} of this tissue may be taken as 2. Consequently this amount of stored oxygen would be used up in 3.4 min. Small amounts of haemoglobin can serve as reservoirs of oxygen over such short periods of time in the case of organisms dependent upon an intermittent supply of oxygen (e.g. *Arenicola marina*, Barcroft & Barcroft, 1924). There is no evidence, however, that the oxygen uptake of nodules is intermittent.

(2) Were the pigment able to move about within the cells, haemoglobin might act as a carrier of

oxygen, but there is no reason to believe that any such movement could occur. It is difficult to see how stationary haemoglobin can have any direct effect upon a diffusion gradient or dissolved oxygen within the nodule after a steady state has been reached.

(3) The presence of haemoglobin might directly affect the rate of combination of oxygen with respiratory enzymes over the range of values of pO_2 at which these enzymes are not saturated with oxygen. This could bring about an increase in the overall uptake of oxygen by the nodule even where this is dependent on the rate of diffusion into the nodular tissue.

If C_o is the external concentration of oxygen, C_s is the concentration of oxygen at a respiratory site, R is the rate of oxygen uptake by enzymes at this site, then, if the enzymes are not saturated with oxygen, the rate of oxygen uptake is nearly proportional to C_s , so that if K is a constant,

$$R = KC_s \quad (1)$$

And if r is the effective length of the diffusion path from the exterior to the respiratory site and D is the diffusion constant

$$R = D(C_o - C_s)/r \quad (2)$$

Combining (1) and (2)

$$R = \frac{C_o}{r/D + 1/K}$$

The possibility under consideration is that the presence of haemoglobin may increase K . This will have an effect upon R , the magnitude of which will depend on the relative magnitudes of $1/K$ and r/D .

This last possibility is apparently supported by certain experimental results. Kubo (1939) found that addition of nodule haemoglobin to *Rhizobium* cells brought about an increase in the rate of respiration at low partial pressures of oxygen. Little & Burris (1947) found a similar effect in the case of *Rhizobium* grown both in the nodule and in pure culture, and a number of other bacteria (*Escherichia coli*, etc.), using a pO_2 of 0.01 atm. Hog haemoglobin had an effect similar to that of the nodule haemoglobin.

The first part of this paper is concerned with diffusion and the oxygen uptake of nodules, the second with the relation of haemoglobin to oxygen uptake—in this section the experiments of Kubo (1939) and of Little & Burris (1947) are discussed.

EXPERIMENTAL METHODS

Nodule material. Legumes were grown in pots containing sterilized soil to which had been added a suspension of cells of the appropriate strain of *Rhizobium*. Sufficient precaution against contaminant infection was obtained by placing plants inoculated with different strains of *Rhizobium* in separate parts of the glasshouse. (Groups of forty plants of each species grown in uninoculated soil simultaneously within the same glasshouse produced no nodules.) Nodules

were normally harvested when the plants were just beginning to flower. They were detached from the plant, washed thoroughly, surface moisture was removed with filter paper, and the nodules were weighed.

Rhizobium suspensions Strains of *Rhizobium* were grown in Roux flasks at 30° on agar medium (K_2HPO_4 , 0.5 g, $MgSO_4 \cdot 7H_2O$, 0.2 g, NaCl, 0.2 g, $CaCl_2$, 0.2 g, $FeCl_3$, 0.001 g, Difco yeast, 5 g, agar, 15 g, distilled water to 1 l) in which a yeast extract preparation was the sole source of carbon and nitrogen. With this medium gum production, which causes inconvenience in the handling of the suspensions, was reduced to a minimum.

Crystalline horse haemoglobin A solution of lyzed horse red blood corpuscles was shaken with ether and centrifuged. The lower layer of oxyhaemoglobin solution was sucked off and dialyzed. Ethanol was added to give a concentration of 20% and the solution left in the ice chest until the haemoglobin had crystallized. Before use the haemoglobin was dialyzed to remove ethanol.

Measurement of the oxygen uptake of detached nodules O_2 uptake was measured in Barcroft differential manometers at 28°. The plant material was usually suspended in 3 ml of Medium 1. CO_2 was absorbed by KOH papers in the centre wells of the flasks. All volumes are expressed as μL of gas at N.T.P.

Medium 1 was as follows: K_2HPO_4 , 0.8 g, KH_2PO_4 , 0.2 g, NaCl, 0.2 g, $MgSO_4 \cdot 7H_2O$, 0.2 g, $CaSO_4 \cdot 2H_2O$, 0.1 g, $Fe_2(SO_4)_3 \cdot 9H_2O$, 0.01 g, glucose, 10 g, distilled water to 1 l, pH 7.3.

RESULTS

Diffusion and the oxygen uptake of nodules

The path of gas uptake in nodules While previous authors (Allison *et al.* 1940) appear to have assumed that gases enter the nodule by diffusion in aqueous solution through the wet cell walls, there is also the possibility that small pores may exist in the outermost layer of cells communicating with the intercellular spaces within the nodule. Gases could then diffuse in directly. In nature nodules are not normally submerged in fluid, but are either more or less dry on the surface or coated with a thin film of moisture. If pores existed in the outermost layer, entry of gases would then be much more rapid under natural conditions than when the nodule was immersed in a fluid in equilibrium with the atmosphere. It is therefore necessary to know how gases do in fact enter the nodule in order to interpret data obtained from experiments on nodules immersed in fluid in manometers.

Sections of nodules of various sizes were examined. A small number of air spaces were seen within the nodule, but none of these appeared to communicate with the exterior. Surface strips of nodules did not reveal any pores or gaps in the outer layer of cells.

Manometric measurements were made of the oxygen uptake of nodules totally immersed in water, and of the same nodules when directly in contact with the gas phase. For this purpose nodules were placed in Barcroft manometers together

with a small amount of water (0.2 ml) which prevented spurious readings being caused by changes in the vapour pressure within the flasks due to absorption of water vapour by the alkali in the centre well. These nodules were in direct contact with air in the manometer flasks. Readings were taken for 30 min, after which 3 ml of water were added to each flask and readings were taken of the O_2 uptake of the nodules when submerged. The manometric constant was recalculated to allow for the relative change in liquid and gas volumes. It had been previously established that the O_2 uptake of nodules, when kept under constant conditions, did not vary over a period of at least 60 min. Measurements on four samples of small soya nodules gave values of 1.67, 1.60, 1.45 and 1.54 for the ratio O_2 uptake of submerged nodules/ O_2 uptake of nodules directly in air, with a mean of 1.55.

This increase in oxygen uptake, observed when nodules were immersed in a fluid shaken so as to be in equilibrium with the atmosphere, was not to be expected if gases are able to diffuse directly through pores into the nodule. The observed effect may perhaps be explained by the small surface available for exchange of gas between gas and liquid phase at the surface of nodules suspended in air, as compared with that at a constantly changing surface of shaking fluid.

It may thus be concluded that gases enter the nodule by passage in solution across the wet walls of the outer layer of cells. While rates of oxygen uptake of nodules measured when immersed in water are somewhat greater than natural rates, they correspond to a known pO_2 at the surface of the nodule.

The Q_{O_2} of nodules of different sizes and of nodule slices From some experiments, in which the rates of oxygen uptake of various nodules and nodule slices were measured, it was possible to obtain an estimate of the extent to which gas uptake of nodules is determined by diffusion.

The oxygen uptake of soya nodules (*Rhizobium*, strain 505) was followed manometrically in air and in oxygen at 28°. After washing and drying, the nodules were graded by size into three samples, small, medium and large, and suspended in medium 1. The values obtained are given in Table 1.

Table 1 *Rates of oxygen uptake of soya nodules of various sizes (Rhizobium, strain 505)*

(The values of mean volume, radius and surface area were calculated from the weight of fresh nodule (density = 1). About 50 nodules of each size were taken.)

	Small nodules	Medium sized nodules	Large nodules
Mean volume of nodule (cu. mm)	2.99	6.38	10.1
Mean radius of nodule (mm)	0.895	1.15	1.35
Mean surface area of nodule (sq. mm)	10.1	16.6	22.9
Q_{O_2} in air	1.26	1.05	0.99
Q_{O_2} in oxygen	3.29	2.82	2.65
			38

Similarly, the Q_{O_2} of whole soya nodules (strain 505) and that of nodule slices of various thicknesses were measured at 28° (Table 2). Nodules were sliced with a razor and the slices allowed to stand in distilled water for about 20 min before the experiment. During the experimental period (about 20 min) the rates remained constant showing no indication of falling off. Thus, together with the fact that the slices were previously thoroughly washed with water, discounts the possibility that the high Q_{O_2} values of the thin slices of tissue were due to substances liberated from the cells on cutting the tissue. Such high values of Q_{O_2} are not uncommon with plant tissues such as young roots.

Table 2 Q_{O_2} of whole nodules and nodule slices

(Soya nodules, *Rhizobium*, strain 505. The same nodule sample was used for experiments in air and in oxygen.)

	Whole nodules	Nodule slices (1 mm thick)	Nodule slices (<0.3 mm thick)
Q_{O_2} in air	1.32	3.59	7.01
Q_{O_2} in oxygen	3.56	8.25	7.76

The enzymes concerned in the uptake of oxygen are saturated at a pO_2 far below 0.2 atm, so that an increase brought about by increasing the pO_2 above this value shows that diffusion is to some extent determining the rate of oxygen uptake. Only in the case of nodule slices less than 0.3 mm in thickness is there practically no such effect on increasing the pO_2 . The value for the Q_{O_2} of the nodule tissue, when independent of the rate of diffusion, may thus be taken as 7–8. Whole nodules have values very much less than this, and furthermore the value of Q_{O_2} decreases as the size of the nodule increases (and thus the ratio surface area/volume decreases). It must be concluded that the rate of uptake is largely determined by diffusion of oxygen within the nodule.

The presence of haemoglobin and oxygen uptake

Two methods of approach were used to find out whether haemoglobin takes any part in oxygen uptake of nodules by virtue of its oxygen-binding capacity. First the rates of oxygen uptake by effective nodules and ineffective nodules (which contain no haemoglobin) were compared, and secondly the effect of carbon monoxide upon oxygen uptake by effective nodules (both detached and attached to the plant) was investigated.

The oxygen uptake of effective and ineffective nodules
The rates of oxygen uptake of detached nodules produced by the effective *Rhizobium* strain 505, and of nodules produced by the ineffective *Rhizobium* strain 507, were measured manometrically at 30°

The values of Q_{O_2} of thin slices of these two types of nodule were also compared (Table 3). The whole nodules were selected so as to be of uniform size. Plants bearing the two types of nodule were grown under identical conditions.

Table 3 *Oxygen uptake of effective and ineffective nodules*

Material	Medium	Oxygen uptake	
		Q_{O_2} in air	Q_{O_2} in oxygen
Whole effective nodules	0.05M phosphate buffer, pH 7.3 + 1% glucose	1.50	—
Whole ineffective nodules		1.69	—
Sliced effective nodules (<0.3 mm thick)	Medium 1	—	7.76
Sliced ineffective nodules (<0.3 mm thick)		—	6.84

The rates of oxygen uptake of whole effective and ineffective nodules, when expressed on a dry weight basis, are equal within the limits of error due to varying sizes of nodule. (This is also true when the results are expressed on a fresh weight basis.) The rates of oxygen uptake by thin slices of these two types of nodule in oxygen (where diffusion of gases within the tissue played no part in determining the rate) did not differ greatly, that of ineffective nodule slices being slightly lower. This result is in contrast to that reported by Asprey & Bond (1941), who found values of Q_{O_2} of whole effective soya nodules (strain 505) to vary between 2.31 and 6.25, while that of ineffective soya nodules (strain 507) was only 0.7. It may be that their ineffective nodules were older, and in a more advanced state of disorganization. In so far as effective and ineffective nodular tissues are comparable, the results (Table 3) show no indication that the haemoglobin in effective nodules affects oxygen uptake.

Carbon monoxide and the oxygen uptake of nodules
By converting all or most of the nodule haemoglobin (Hb) to carboxyhaemoglobin (HbCO) its property of reversible oxygenation is removed, and from the resulting rate of respiration it can be seen whether this property plays any part in determining the normal oxygen uptake of nodules.

The maximum partial pressure of carbon monoxide (pCO) necessary to obtain a ratio HbCO/total Hb equal to 0.5 may be approximately calculated from data given by Keilm & Wang (1945)

In the equilibrium $HbO_2 \rightleftharpoons Hb + O_2$

$$K_1 = [Hb] pO_2 / [HbO_2]$$

In the equilibrium $HbCO \rightleftharpoons Hb + CO$

$$K_2 = [Hb] pCO / [HbCO]$$

If C is the total concentration of haemoglobin in all forms

$$C = [\text{Hb}] + [\text{HbO}_2] + [\text{HbCO}]$$

When $[\text{HbCO}]/C = 0.5$

$$p\text{CO} = K_2 + \frac{K_2}{K_1} p\text{O}_2$$

From Keilin & Wang's data

$$K_1 = 0.1 \text{ mm Hg,}$$

$$K_2 = 0.0027 \text{ mm Hg}$$

According to Keilin & Wang K_1 may be less than 0.1 mm but the difference does not alter the calculated value of $p\text{CO}$ to any significant extent

Taking the maximum value of $p\text{O}_2$ which is found at the surface of the nodule in air $p\text{O}_2 = 152$ mm mercury, the maximum value of $p\text{CO}$ is calculated to be 0.0055 atm (0.55 % CO). With 5 % CO practically all the haemoglobin would be in the form HbCO

The effects of these concentrations of carbon monoxide (which are below those affecting cytochrome oxidase or other respiratory enzymes) on the oxygen uptake of excised nodules and of nodules attached to the plant were studied

The effect of carbon monoxide on the oxygen uptake of detached nodules Whole nodules, and in some experiments sliced nodules and roots for comparative purposes, were suspended in Medium 1 in Barcroft

Table 4 *Oxygen uptake of whole soya nodules (Rhizobium, strain 505)*

(q_{O_2} is the oxygen uptake expressed as $\mu\text{l/mg}$ of fresh tissue/hr Temp 28°)

q_{O_2} in air	0.134	0.155
q_{O_2} in air + CO (0.0055 atm)	0.172	0.172

Table 5 *Oxygen uptake of Vicia faba nodules (effective Rhizobium, strain 2195) sliced nodules and roots 30°*

	Whole nodules	Whole nodules	Sliced nodules	Roots	Roots
Rate of O_2 uptake in air ($\mu\text{l/hr}$)	80	117	150	102	144
Rate of O_2 uptake in 20% O_2 , 5% CO , 75% N_2 ($\mu\text{l/hr}$)	80	122	156	84.6	123

manometers at 28 or 30° , and their rate of oxygen uptake measured in air. The flasks were filled with gas mixtures, containing 20 % oxygen in nitrogen with varying concentrations of carbon monoxide, and readings continued. Readings in the gas mixture containing carbon monoxide were continued over 40 min (Tables 4 and 5)

Carbon monoxide in concentrations up to 5 %, when all the Hb would be converted into HbCO , had

no inhibitory effect on oxygen uptake by detached nodules (In fact there was often a slight accelerating effect. This will be discussed later, together with some experiments on bird red blood cells)

Carbon monoxide and the oxygen uptake of nodules attached to the plant

A striking and at present inexplicable fact of symbiotic nitrogen fixation is that, after their separation from the rest of the plant, nodules and nodulated roots lose all but a trace of their nitrogen-fixing ability. Nodules when excised are clearly different in some way from those still attached to the plant. It was thought that this difference might be reflected in the response of their oxygen uptake to poisoning of the haemoglobin with carbon monoxide. With this end in view the experiments of the last section were repeated using intact nodulated plants.

Experimental method While Bond (1939) has measured the CO_2 output of nodulated roots attached to the plant, similar measurements of O_2 uptake cannot be traced in the literature

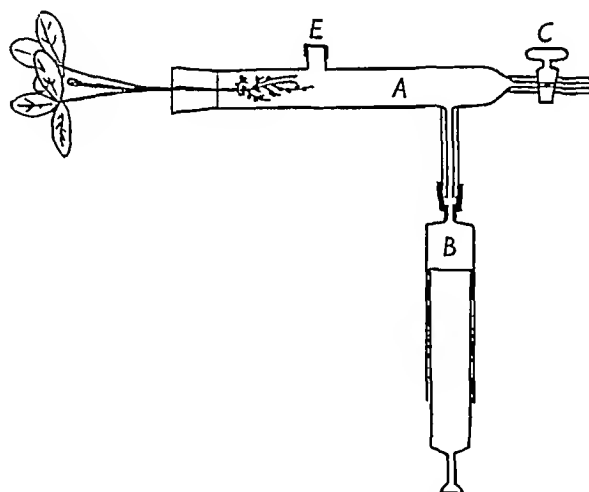


Fig 1 Apparatus used in the measurement of oxygen uptake of nodulated roots attached to the plant

For this purpose the simple apparatus (Fig 1) was devised. The plant was held tightly in the apparatus by means of the split-bored rubber bung, so that the nodulated roots projected into the cylindrical glass vessel A (volume, about 20 ml). This vessel, together with the syringe B (connected by a glass capillary and rubber tube), was filled with nutrient solution saturated with a gas mixture, and all gas bubbles were expelled through tap C which was then closed. There was then no gas phase in contact with the fluid surrounding the roots. The apparatus was attached to a holder which fitted into the shaking mechanism of the manometer bath. By this means the vessel A was given a horizontal oscillatory movement so that the fluid within was adequately stirred by a number of large rolling glass beads placed in A. Fluid in B was not in equilibrium with that in A owing to the narrow capillary connecting A and B.

From time to time samples of the fluid in *A* were removed through a short piece of sealed rubber tubing *E* into a syringe, devised by Roughton & Scholander (1943), the capillary end of which had been cemented to a no. 20 hypodermic needle (Fig. 2). As the sample was removed from *A* it was replaced by an equal volume of fluid, containing the initial amount of dissolved gas, from the syringe *B*. (The plunger of this syringe was kept under a slight positive pressure by a rubber band.) Allowance was made for this replacement in the calculation of the results. (Transpiration by the small

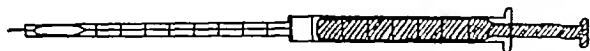


Fig. 2 Modified Roughton-Scholander syringe

clover plants used was negligible over the period of the experiment.) The dissolved gases in the sample (about 0.4 ml fluid) were extracted with CO_2 in the main part of the syringe, the CO_2 absorbed with KOH , and the bubble of extracted O_2 and N_2 pushed into the capillary of the syringe where its volume was measured (after immersion of the syringe in a bath of water at room temperature). The O_2 was then absorbed by alkaline $\text{Na}_2\text{S}_2\text{O}_4$ and the bubble of N_2 measured. This analytical procedure is described by Roughton & Scholander (1943). During the shaking operations the needle was closed with a small piece of spongy rubber.

During the course of these experiments the $p\text{O}_2$ was constantly changing, as no provision was made for the replacement of the O_2 used. It was found experimentally that, above a certain value, changes in $p\text{O}_2$ did not affect the rate of O_2 uptake by nodulated roots of small clover plants. (This would not be true of thick roots owing to diffusion

Values of the rate of oxygen uptake of pieces of plant tissue measured in the apparatus and in Barcroft manometers agreed to within 2%.

Experiments Those roots bearing few or no nodules were removed from a well nodulated clover plant (inoculated with *Rhizobium*, strain 2192). The latter was then washed thoroughly and placed in the apparatus filled with Medium 1 equilibrated with a mixture containing 20% N_2 and 80% O_2 . The apparatus was shaken and readings of the O_2 content of the fluid taken. After about 90 min tap *C* was opened, and 0.4 ml of medium 1 saturated with CO was introduced through *E* by means of a syringe with a needle. This displaced an equal volume of medium through tap *C*. By this means a $p\text{CO}$ of 0.02 atm was obtained. Tap *C* was then closed and readings were continued.

Several experiments of this kind were carried out, typical results are shown in Fig. 3. No effect of carbon monoxide at this partial pressure on the oxygen uptake rate could be detected. Any change in rate of oxygen uptake of about 5% or more would easily have been observed.

Haemoglobin and the oxygen uptake of the root nodule bacteria

Mention has already been made of the experiments of Little & Burris (1947), who found that addition of mammalian or nodule haemoglobin to resting suspensions of *Rhizobium* brought about an increase in the rate of oxygen uptake at low partial pressures of oxygen (0.01 atm). Their interpretation of this result as a direct effect of haemoglobin on the oxygen uptake of the nodule bacteria, the pigment acting through its capacity for reversible oxygenation, made it likely that the same effect might be present in the nodule. From the experiments with effective and ineffective nodules, and with carbon monoxide, described above, it is apparent that this is not so. In order to learn if their results could be interpreted in another way the experiments of Little & Burris (1947) were repeated at values of $p\text{O}_2$ of 0.01 and 0.2 atm with carboxyhaemoglobin as a control in addition to denatured haemoglobin.

Experiment 1 A 4-day culture of *Rhizobium* (strain 2193) grown at 30° was washed and suspended in 0.1 M phosphate buffer pH 7.3. Crystalline haemoglobin, prepared from horse red blood cells as described (p. 593), was dissolved in 0.1 M phosphate buffer pH 7.3. Denatured haemoglobin was obtained by heating this solution to 100° for 1 min (following the procedure of Little & Burris, 1947). O_2 uptake was measured manometrically at 37.2° in atmospheres of O_2 of partial pressure 0.01 atm or 0.2 atm in N_2 . In each flask was placed 1 ml of the *Rhizobium* suspension, 1 ml of 3% glucose, together with either 1 ml of buffer solution, 1 ml of the haemoglobin solution in buffer, or 1 ml of denatured haemoglobin.

Table 6 shows that the effect of haemoglobin is large at both values of $p\text{O}_2$. The haemoglobin denatured by heating inhibited oxygen uptake to some

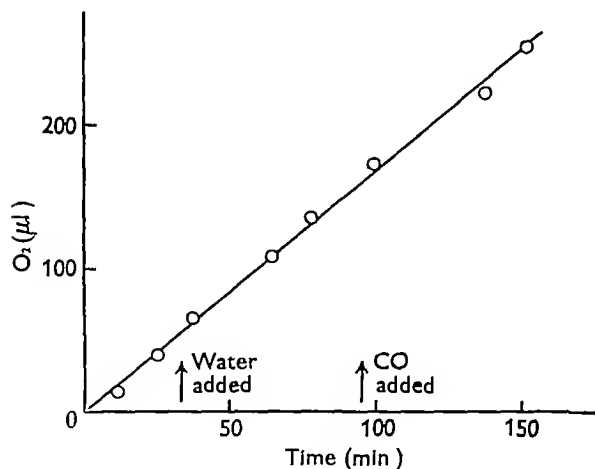


Fig. 3 Oxygen uptake of nodulated clover roots attached to the plant, with and without CO ($p\text{CO} = 0.02$ atm)

effects.) It was found convenient to fill the apparatus at the start of an experiment with Medium 1 (without glucose) approximately saturated with a gas mixture containing 80% O_2 and 20% N_2 . In filling the apparatus this was first washed with the gas mixture, passed through via the sampling tube *E*, and then the medium saturated with the gas mixture was passed through. The tube *E* was then closed by the piece of sealed rubber tubing and any gas bubbles expelled through tap *C*.

extent and was unsatisfactory as a control, consequently in the next experiment it was replaced by carboxyhaemoglobin

Table 6 Haemoglobin and oxygen uptake of Rhizobium

Contents of manometer flask	Rhizobium (suspension in glucose buffer + hae moglobin)	Rhizobium (suspension in glucose buffer + buffer)	Rhizobium (suspension in glucose buffer + de-natured hae-moglobin)
Rate of O ₂ uptake (μl/hr) at pO ₂ =0.01 atm	49.5	23.4	15
Rate of O ₂ uptake (μl/hr) at pO ₂ =0.2 atm	53.5	39.6	21

The oxygen capacity of the haemoglobin solution added, determined manometrically by the ferricyanide method, was 29.2 μl O₂/ml solution

Increasing the rate of shaking of the flasks did not affect the rates of oxygen uptake

Experiment 2 The first experiment was repeated with some flasks filled with air and others with a gas mixture containing 20% O₂, 5% CO and 75% N₂. Haemoglobin in flasks filled with the CO mixture was entirely converted to HbCO

The oxygen-uptake curves (Fig. 4) showed that, in addition to haemoglobin, carboxyhaemoglobin brought about equally large increase in the oxygen

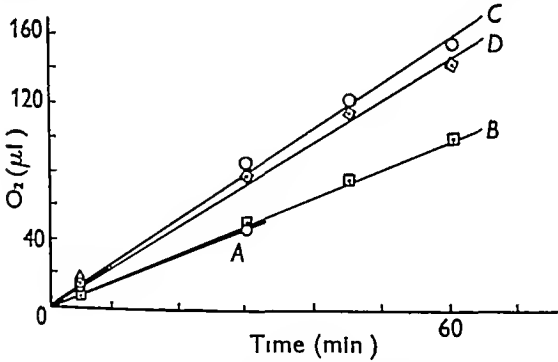


Fig 4 Oxygen uptake of Rhizobium suspensions A, in air, B, in air containing CO, C, in air, with haemoglobin, D, in air containing CO, with haemoglobin

uptake of the bacteria. The effect of haemoglobin in this and probably in the other experiments, including those at low partial pressures of oxygen, is not connected with its ability to undergo oxygenation. A probable explanation is that Rhizobium and many other bacteria are able to break down haemoglobin and use it as a nitrogen source, so that in the presence of haemoglobin, the bacteria change over from a rate of oxygen uptake corresponding to a resting metabolism to a higher rate characteristic of a proliferating metabolism. A similar effect of haemoglobin on bacterial respiration at low values of pO₂, noted

by Baumberger (1939), may possibly be interpreted in the same way

Haemoglobin and oxygen uptake in bird red blood cells The nucleated red blood corpuscle of birds provides a model cell containing a large quantity of haemoglobin and having a reasonably high respiratory activity. Warburg (1929) has used these cells in an experiment intended to find out whether stationary haemoglobin within cells can have any effect on their oxygen uptake. He measured the change in oxygen uptake of bird red blood cells on converting all their haemoglobin to carboxyhaemoglobin, and found the unexpected result that treatment of these cells with carbon monoxide caused an increase in their oxygen uptake. Warburg does not explain this odd effect satisfactorily and gives no details of his experiments.

His experiment has been repeated here using fresh washed chick red blood corpuscles suspended at 37° in bird Ringer solution containing 0.2% glucose (Bird Ringer solution: NaCl, 9 g, KCl, 0.4 g, CaCl₂, 0.25 g, water to 1 l). Into each of a number of Warburg manometers were placed 2 ml of this suspension. Oxygen uptake was measured in air and in a gas mixture containing 5% carbon monoxide, 20% oxygen and 75% nitrogen.

Immediately after replacing the air in the flasks with the gas mixture containing carbon monoxide the effect noted by Warburg was observed, the oxygen uptake increasing by more than 50%. However, after about 20 min the rate fell quickly back to the original value obtained in air (Table 7).

Table 7 Oxygen uptake of bird red blood corpuscles in air and in air containing 5% carbon monoxide

(Each flask contained 2 ml corpuscles. O₂ capacity of the haemoglobin in 2 ml corpuscles = 133 μl. Temp., 37°)

	Rate of O ₂ uptake (μl/hr)
In air	68.4
Initial rates in air containing 5% CO	99.6, 91.2*
Final rate in air containing 5% CO	67.2, 66.6*

* Duplicate determinations

DISCUSSION

While in most cases the occurrence of haemoglobin can be directly related to its function as an oxygen carrier, some organisms (including some invertebrates and certain protozoa) possess a stationary intracellular haemoglobin, which cannot possibly act as an oxygen carrier in the normal sense and yet which has the same property of reversible oxygenation. This is also true of the bacteria containing cells of the legume nodule. Here, in addition, there is good evidence that the presence of the pigment is in some way connected with the process of symbiotic nitrogen fixation.

The view that nodule haemoglobin acts in nitrogen fixation as an oxidation-reduction catalyst, the valency of the iron in the molecule undergoing reversible changes, must be rejected both on experi-

mental and theoretical grounds (Keilm & Smith, 1947) It has often been assumed that such stationary haemoglobin might bring about an increase in the oxygen uptake of cells, especially when these are in an environment deficient in oxygen In face of the known interrelation between oxygen supply and the functioning of the legume nodule such an explanation of the presence of haemoglobin in the nodule seemed not unlikely This was more especially so in view of the low pO_2 in the central nodular tissue

It was therefore necessary to compare the oxygen uptake of nodules with and without haemoglobin capable of reversible oxygenation This was done in two ways and in each case no significant difference in oxygen uptake was detected The first comparison, that between effective and ineffective nodules is open to the objection that ineffective nodules differ from effective nodules not only because of their lack of haemoglobin, but also because of the breakdown of the bacteria-containing cells Such criticisms do not apply, however, to the comparison between normal nodules and nodules in which the haemoglobin has been converted to carboxyhaemoglobin Within the accuracy of the methods used in measuring oxygen uptake (approx $\pm 2\%$) the presence of haemoglobin has no effect on the oxygen uptake of nodules whether excised or attached to the rest of the plant Such a conclusion is compatible with the results found on examining the effect of solutions of haemoglobin upon the oxygen uptake of *Rhizobium* suspensions The increase in oxygen uptake of *Rhizobium* suspensions on addition of haemoglobin solutions was found not to be connected with the reversible oxygenation property of haemoglobin, but to be probably due to the ability of the bacteria to use haemoglobin as a nitrogen source

Because of its comparatively high respiratory activity and its high haemoglobin content, the nucleated bird red blood cell appeared to be an ideal model on which to investigate the effect of haemoglobin contained within the cell on its oxygen uptake However, low concentrations of carbon monoxide bring about a temporary increase of about 50% in the oxygen uptake of these cells It is possible that this increase in gas uptake is in reality due to the

combustion of carbon monoxide to carbon dioxide which, as has been shown by Fenn & Cobb (1932), may take place in frog muscles It was thus not possible in these cells to find the effect on oxygen uptake of converting the haemoglobin to carboxyhaemoglobin

SUMMARY

1 It is shown that oxygen enters the nodules of leguminous roots by diffusion in solution across the wet cell walls

2 Values of the Q_{O_2} of whole nodules decrease with increasing size of nodule and are greater in oxygen than in air The Q_{O_2} of thin slices of nodules, of a thickness such that diffusion does not determine the rate of oxygen uptake, is about 7–8 $\mu\text{l}/\text{mg}$ dry wt/hr, while that for whole nodules is about 1–2 $\mu\text{l}/\text{mg}$ dry wt/hr

3 The Q_{O_2} values of whole and sliced ineffective nodules differed very little from those of whole and sliced effective nodules

4 Carbon monoxide in a concentration sufficient to convert practically all the nodule haemoglobin to carboxyhaemoglobin had no effect on oxygen uptake by detached effective nodules An apparatus is described by means of which the oxygen uptake of nodulated roots may be measured when these are attached to the plant No effect of low concentrations of carbon monoxide on the oxygen uptake of attached nodules could be detected

5 The increase in oxygen uptake brought about by addition of haemoglobin solutions to *Rhizobium* cells is shown to be unconnected with the ability of haemoglobin to undergo reversible oxygenation

6 Warburg's (1929) observation that low concentrations of carbon monoxide apparently stimulated oxygen uptake by bird red blood cells was confirmed It was found that this stimulation was temporary

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The Inhibition of β -Glucuronidase by Saccharic Acid and the Role of the Enzyme in Glucuronide Synthesis

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In view of the relationship shown to exist between the β glucuronidase activity of a tissue and its state of growth (Levy, Kerr & Campbell, 1948), it was considered important to find a specific inhibitor for this enzyme. It has been suggested (Fishman, 1940), without any direct evidence, that β glucuronidase is responsible for the formation of glucuronides in the body. The use of an inhibitor for glucuronidase in testing this hypothesis forms an obvious first step towards elucidating the physiological function of the enzyme.

A variety of substances have been examined for their effect on the hydrolysis of phenylglucuronide

by β glucuronidase. Of those which caused inhibition, by far the most effective was D-glucosaccharic acid, and this compound was examined for its action on glucuronide synthesis by liver slices and on growth processes in the mouse.

EXPERIMENTS AND RESULTS

Determination of β glucuronidase The hydrolysis of phenylglucuronide by mouse-liver or kidney glucuronidase preparations was measured by the procedure of Kerr, Graham & Levy (1948). In testing substances for a possible inhibitory action on the enzyme, incubation mixtures were made up as follows: 0.4 ml enzyme preparation, 0.2 ml

Table 1 *Inhibition of β -glucuronidase in vitro (0.015M-phenylglucuronide)*

Compound	Concentration (10^{-4} M)	Phenol liberated		Inhibition (%)	Enzyme preparation
		In controls (μ g)	In presence of inhibitor (μ g)		
Saccharic acid	150	32.1	3.3	90	Liver
	150	25.4	4.2	84	"
	150	17.1	3.2	81	"
	50	39.1	8.0	80	"
	50	32.7	6.5	80	"
	50	27.0	6.5	76	"
	50	25.5	7.9	69	"
	50	21.2	3.2	85	"
	50	16.5	5.8	65	"
	50	20.5	4.6	78	Liver A
	50	22.8	3.4	85	Liver B
	50	22.5	0.5	98	Crude liver
	50	11.8	3.8	68	Kidney
	50	21.2	6.5	70	"
	50	17.0	5.7	67	Crude kidney
Mucic acid	75	30.2	23.6	22	Liver
D Gluconic acid	150	37.4	28.8	23	"
D Glucuronic*	300	40.0	0	100	"
	150	35.3	12.1	66	"
	38	55.2	32.4	41	"
	10	55.2	45.2	18	"
	3.3	55.2	51.2	7	"
L Malic acid†	150	23.7	14.9	37	"
DL-Malic acid	300	32.9	18.6	44	"
	150	30.2	23.5	22	"
Phlorrhizin‡	3	16.3	13.6	17	"
	1.5	16.3	16.8	-3	"
Vanillin‡	7.5	39.4	28.8	27	"

* Interferes in colour reaction for phenol. Results are corrected for interference.

† The naturally occurring isomer, commonly called laevorotatory malic acid.

‡ Gives colour with phenol reagent. Results are corrected for this colour.

0.1 M-citrate buffer, 0.1 ml 0.12 M-phenylglucuronide, 0.1 ml inhibitor solution. In controls, water was substituted for the inhibitor solution. Buffer, substrate and inhibitor solutions were, as a rule, adjusted to pH 5.2 (glass electrode). In experiments in which the two glucuronidase fractions in mouse liver were separated (Kerr, Campbell & Levy, 1949, Mills, 1948), however, hydrolysis with fraction A was carried out at pH 4.5 instead of 5.2. Occasionally, preliminary purification of the enzyme was omitted, and the crude liver or kidney homogenate was used for hydrolysis. Results are expressed as μg of phenol liberated in 1 hr at 37° .

Measurement of glucuronide synthesis The conjugation of *o*-aminophenol with glucuronic acid was followed by the method of Levy & Storey (1949). After removal of protein with a mixture of trichloroacetic acid and phosphate buffer, the glucuronide was diazotized and coupled with naphthylethylenediamine. At the pH selected for colour development, free *o*-aminophenol in comparatively large amounts did not interfere. To measure the synthetic activity of mouse liver slices, they were shaken in sulphate-free bi-

a fall in the activity of the enzyme are listed in Table 1. The most effective was D-glucosaccharic acid, and Fig. 1 shows the percentage inhibition produced by varying concentrations of this compound in three experiments with liver glucuronidase. It can be seen that 50% inhibition was obtained with 2×10^{-4} M-saccharate, and practically complete inhibition with less than 10^{-2} M (substrate concentration 0.015 M). From results given in Table 1, it appears that the inhibitory action of saccharate was independent of the following factors: the source of the enzyme, the activity of the preparation, the glucuronidase fraction present, and the degree of purity of the preparation.

Of other compounds listed in Table 1, three were closely related to saccharic acid (mucic, gluconic and glucuronic acids), but were much less efficient as inhibitors of β -glucuronidase. Glucuronic acid

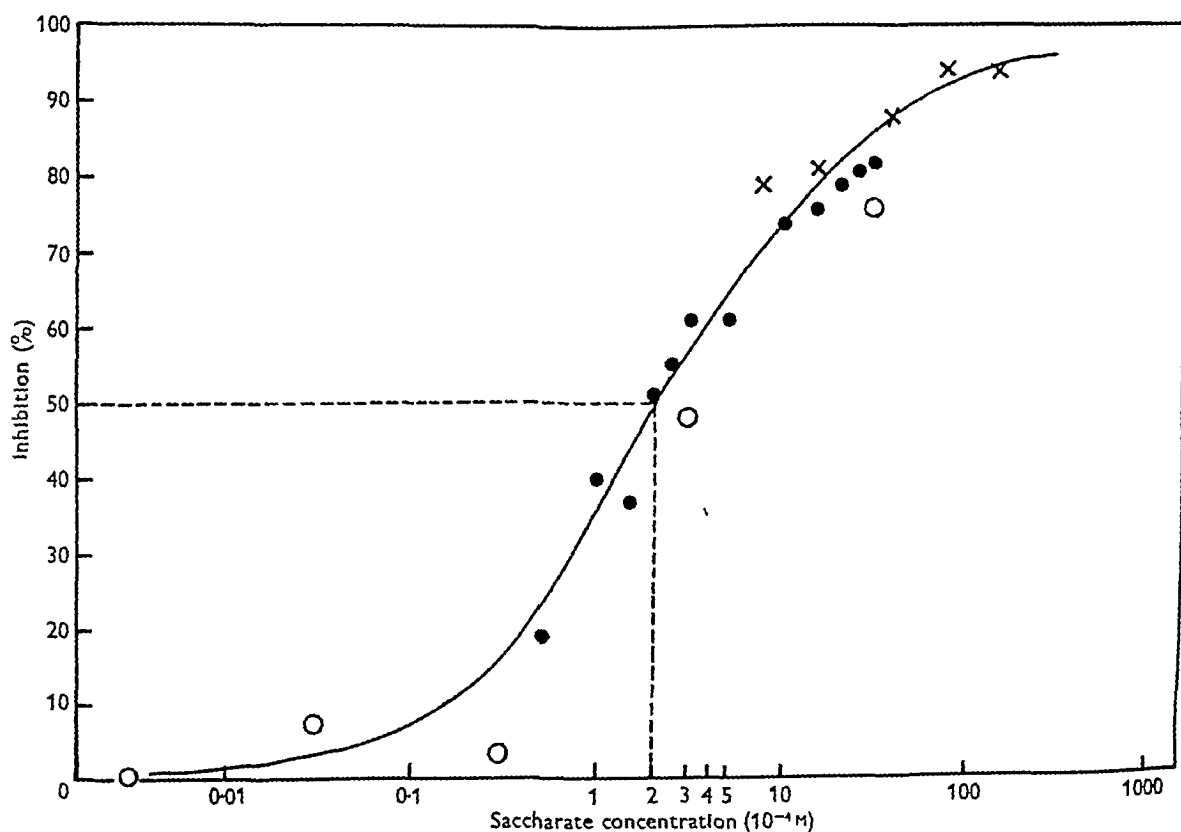


Fig. 1. Effect of varying concentrations of saccharic acid on the hydrolysis of phenylglucuronide (0.015 M) by mouse liver glucuronidase (results for three separate experiments shown by O, ● and ×).

carbonate Ringer solution, containing 0.02 M lactate, 0.001 M ascorbic acid and 0.0025% *o*-aminophenol, at 37° for 1 hr in an atmosphere of 5% CO_2 in O_2 . Results are expressed as μg *o*-aminophenol conjugated/g dry wt of tissue in 1 hr.

Inhibition of β -glucuronidase in vitro

Nearly fifty substances were examined for their effect on β -glucuronidase *in vitro*. Those which caused

(D-glucurone) interfered in the determination of phenol liberated from phenylglucuronide. In the absence of phenol, glucuronic acid was without effect on the Folin-Ciocalteu reagent, but in the presence of phenol it apparently gave the colour reaction. This effect was independent of the phenol concentration. The figures shown in Table 1 for the inhibitory action of glucuronic acid on glucuronidase have been corrected for interference in the colour reaction, and

are considered reliable. Correction of the hydrolysis figures was also necessary in the case of phlorrhizin and vanillin which gave the colour reaction directly. After correction, the results suggested that both compounds slightly inhibited β -glucuronidase. L-Malic acid in high concentration had an inhibitory action on the enzyme which entirely accounted for the effects produced by the racemic acid. A comparative study of the tartaric acids might provide interesting information regarding configurational requirements for glucuronidase inhibition. Unfortunately, only L-tartaric acid* was available, and this had no effect on the enzyme (see below).

The following substances had no apparent effect on the hydrolysis of phenylglucuronide by β glucuronidase in the concentrations shown: β phenyl D glucoside (0.015 M), α methyl D glucoside (0.015 M), α methyl D mannoside (0.003 M), β methyl D glucoside (0.003 M), β methyl D xyloside (0.003 M), α methyl D galactoside (0.003 M), gum arabic (0.15%), degraded egg plum gum (0.05%), pyromucic acid (0.015 M), sorbic acid (0.02 M), oxalic acid (0.015 M), malonic acid (0.015 M), succinic acid (0.015 M), glutaric acid (0.015 M), maleic acid (0.015 M), L tartaric acid (0.015 M), valeric acid (0.015 M), ouabain (0.0015 M), digitonin (0.0015 M), urethane (0.015 M), phenylurethane (0.015 M), nitroso N-methylurethane (0.015 M), heparin (6.6 Toronto units/ml), sulphapyridine (0.00015 M), inositol (0.015 M), piperonal (0.015 M), n hexyl alcohol (0.015 M), NN di (2 chloro ethyl) aniline (0.02 M), 2' methyl 4 dimethylaminostilbene (0.01 M), NaF (0.015 M), Na_2SO_4 (0.03 M).

The following compounds gave colours with the phenol reagent, but, when correction was made for this, they were apparently without effect on β glucuronidase in the concentrations shown: salicin (0.015 M), thiourea (0.0015 M), eserine (0.0001 M), ascorbic acid (0.00075 M), oestrone (0.0005 M), colchicine (0.0001 M).

The following compounds interfered too badly in the colour reaction to be tested with β glucuronidase: sodium azide, ω bromoacetophenone, phenylarsenoxide, ethylecyanoacetate, ethane 1,2 dithiol.

The action of saccharic acid on β -glucuronidase

From its similarity in structure to glucuronic acid, one would expect saccharic acid to act competitively in inhibiting glucuronidase. That the inhibition was reversible was shown by precipitating the enzyme from 0.03 M saccharate solution with an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. After one precipitation, the activity was 70% of that shown by a control sample of the enzyme. After dissolving in water and reprecipitating, the activity was as great as in the control.

The normal substrate-activity curve for the hydrolysis of phenylglucuronide by mouse-liver glucuronidase has been studied by Kerr *et al* (1948, see also Kerr *et al* 1949). An approximate value of 0.0035 M was obtained for K_m , the concentration

giving half the maximum velocity of hydrolysis. The maximum was usually reached with 0.015 M substrate. Inhibition by excess substrate was pronounced.

Fig 2 shows the effect of increasing the concentration of phenylglucuronide on its initial rate of hydrolysis in presence of 2×10^{-4} M-saccharate. Results are expressed in terms of the relative activity, where hydrolysis of 0.015 M-phenylglucuronide in absence of inhibitor is taken as unity. The points show averages for two representative experiments, one with liver glucuronidase fraction A and the other with fraction B. There was no appreciable difference in the results for the two fractions. It is clear that

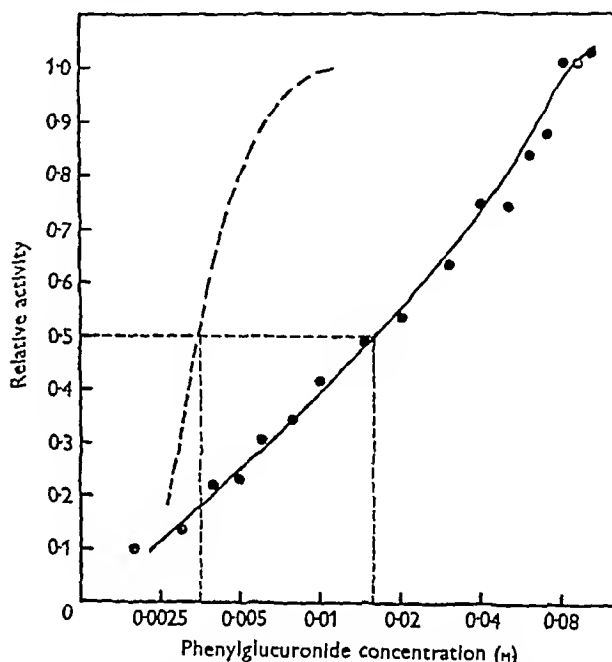


Fig 2 Effect of varying concentrations of phenylglucuronide on its hydrolysis by mouse liver glucuronidase in presence of 2×10^{-4} M saccharate (●—●). Results expressed as fractions of the maximum activity observed in absence of inhibitor. Substrate activity curve in absence of inhibitor (Kerr *et al* 1948) shown by broken line.

saccharate acted competitively since the effect decreased with increasing substrate concentration till at 0.08 M-phenylglucuronide the activity of the enzyme was fully restored.

From Figs 1 and 2, it can be seen that, in presence of a saccharate concentration $[I]$ of 2×10^{-4} M, half the maximum enzyme activity was reached with a substrate concentration of 0.015 M. The latter figure can be designated K'_m , and K_i , the dissociation constant of the enzyme-inhibitor complex, can be calculated from the equation $K_i = [I] K_m / (K'_m - K_m)$ (Lineweaver & Burk, 1934). Using the value for K_m given by Kerr *et al* (1948), $K_i \approx 6 \times 10^{-5}$ M. It should be stressed that, presumably owing to the presence of impurities, K_m can vary from enzyme preparation to preparation by as much as 50% in absence

* The naturally occurring isomer, commonly called dextrorotatory tartaric acid.

of added inhibitor. This is associated with variations in the substrate concentrations at which maximum activity is reached and inhibition by excess substrate becomes marked. The value for K_i is not materially altered since K'_m varies with K_m . Attempts to determine K_i by the method of Hunter & Downs (1945), in which knowledge of K_m is not required, were unsuccessful.

The effect of saccharic acid on glucuronide synthesis by mouse liver slices

Table 2 shows the effect of saccharic acid in varying concentration on the formation of *o*-aminophenylglucuronide by mouse liver slices. Each figure is an average for a determination done in quadruplicate, as recommended by Levy & Storey (1949). These authors found the standard deviation of a single observation from the mean to amount to 17 %.

Table 2 *Action of dicarboxylic acids on glucuronide synthesis by mouse liver slices*

Exp no	Compound	Concentration ($10^{-4}M$)	<i>o</i> Amino-phenol conjugated/g dry wt (μg)	Difference from control (%)
1	Control	—	210	
	Saccharate	8	190	-10
2	Control	—	200	
	Saccharate	8	170	-15
3	Control	—	460	
	Saccharate	50	500	+9
4	Control	—	390	
	Saccharate	50	300	-23
5	Control	—	620	
	Saccharate	50	540	-13
	Saccharate	100	540	-13
6	Control	—	840	
	Saccharate	100	580	-31
	Saccharate	170	550	-34
7	Control	—	340	
	Saccharate	100	270	-20
	Maleate	100	270	-20
8	Control	—	780	
	Saccharate	100	720	-8
	Succinate	100	660	-15

in their procedure. The standard error for a figure based on four results is thus 10 %. Differences in synthetic activity between saccharate-treated slices and control slices from the same animal approached significance in only one experiment (no. 6). Taking the results as a whole, however, synthesis in presence of saccharate tended to be slightly less than in its absence. This effect was non-specific since it was also seen in experiments with succinic and maleic acids. These acids have no action on β -glucuronidase (see p. 601). All three dicarboxylic acids studied were

added as solutions brought to neutrality with potassium hydroxide (glass electrode).

Experiments were done to show that saccharic acid can inhibit hydrolysis of *o*-aminophenyl glucuronide by β -glucuronidase. The final substrate concentration was arbitrarily fixed at 0.13 % and the pH at 4.5 (citrate buffer). After 2 hr incubation at 38° with mouse-liver glucuronidase, 11 % hydrolysis of the glucuronide was observed in absence of saccharate. In presence of $10^{-3}M$ -saccharate, the hydrolysis was 0.7 %. When the experiment was repeated with another enzyme preparation, the hydrolysis in absence of saccharate was 17 % and in its presence 3.6 %.

Penetration of saccharic acid into the cell

Attempts were made to show inhibition of β -glucuronidase in the intact cell by saccharic acid. In the first experiment, mouse-liver slices of known weight from two animals were shaken in sulphate free bicarbonate Ringer solution containing 0.01M saccharate for 90 min at 37°. At the end of this period, the slices were removed, washed in three changes of distilled water, and homogenized. Inactive protein was precipitated by incubation of the homogenate for 30 min at pH 5.2. Without further purification, the supernatant was examined for β -glucuronidase activity. The activity in terms of μg phenol liberated at 37° in 1 hr by 1 g liver was 220, compared with 334 for control slices from the same two animals put through the procedure in absence of saccharate. Unless saccharate was strongly adsorbed on the surface of the slices, it would appear that an appreciable amount penetrated the cells. On the assumption that the saccharate concentration within the slices rose to 0.01M, the inhibition expected was of the order of 75 %.

In other experiments, the enzyme preparation in the incubation mixture used in the assay of glucuronidase activity was replaced by mouse liver slices of known weight. The results had little quantitative value as the 'enzyme blank' was variable and high, but they suggested that some hydrolysis of phenyl glucuronide took place and that this process was strongly inhibited by 0.015M saccharate.

The effect of saccharate on the oxygen uptake and anaerobic glycolysis of mouse liver slices

Saccharate in a concentration of 0.014M had no effect either on the oxygen uptake or on the anaerobic glycolysis of mouse liver slices as measured in the Warburg apparatus. The Ringer solutions of Krebs & Henseleit (1932) were used, the O_2 consumption being determined in phosphate Ringer and an atmosphere of O_2 , and the CO_2 output in bicarbonate Ringer and an atmosphere of 5 % CO_2 in N_2 .

The action of saccharic acid on growth processes in the mouse

A compound that inhibits β -glucuronidase *in vitro* can be hardly expected to arrest whatever mechanism is responsible for the increase in the activity of the enzyme normally observed *in vivo* when a tissue is stimulated to rapid growth. If, however, glucuronidase plays an essential part at some stage in the growth process, administration of an inhibitor might modify the process at that stage. This possibility was examined with saccharic acid. When large doses were administered to mice, saccharic acid was apparently without effect on liver regeneration following damage and on growth in infant mice. Figures for glucuronidase activity, the weights of single organs or of the whole animal, and the histological picture were invariably identical with those observed in appropriate controls. It should be pointed out that during the preparation of the enzyme for assay it would be freed from any saccharic acid which might have been present in the original tissue.

Saccharic acid given at frequent intervals by subcutaneous injection of neutralized solutions of the potassium hydrogen salt in doses totalling up to 5 g/kg daily for periods up to 8 days had no action on liver repair after administration of CCl_4 or partial hepatectomy (Levy & Storey 1948). It failed to modify the increase in uterine weight and glucuronidase activity observed in ovariectomized mice during liver regeneration (Kerr *et al.* 1949). In infant mice, intraperitoneal injection of 2 g saccharic acid/kg daily or the addition of 3% potassium hydrogen saccharate to the solid diet had no effect on normal growth after as long as 3 weeks.

Attempted synthesis of o-aminophenylglucuronide by β -glucuronidase

Florkin, Crismer, Duchateau & Houet (1942) claim to have demonstrated conjugation of borneol (saturated solution) with glucuronic acid (0.01M) in presence of ox spleen glucuronidase. At the end of the incubation period, free glucuronic acid was removed with copper sulphate and calcium hydroxide and glucuronic acid in combination was estimated by the Tollens colour reaction. Only a small fraction of the total glucuronic acid present was in the combined form, even after incubation for several days.

The use of o-aminophenol as the aglycone in demonstrating glucuronide synthesis (Levy & Storey, 1949) has the advantage that in the final reaction traces of the conjugate give a pink colour which is never seen in controls. No formation of o-aminophenylglucuronide was detected in experiments in which the free phenol was incubated with glucuronic acid in the presence of concentrated preparations of mouse liver glucuronidase.

D Glucurone was present in final concentrations varying from 0.4 to 0.0125M in 0.05M citrate buffer at pH 5.2, or 0.05M phosphate buffer at pH 7.4, containing 0.0025% o-aminophenol, 0.001M ascorbic acid, and the enzyme. The mixture was shaken for periods of 2 and 22 hr at 37°. In the longer term experiments, the incubation flasks were filled with N_2 to prevent oxidation of the free phenol.

When liver slices were replaced in the procedure of Levy & Storey (1949) by crude liver homogenate, no glucuronide synthesis was detected.

DISCUSSION

Considerable difficulties were encountered in determining K_i , the dissociation constant for the inhibitor-enzyme complex, in the case of saccharic acid and β -glucuronidase, but it is considered that the value of 6×10^{-5} M finally arrived at is at least as reliable as values quoted for K_m , the dissociation constant of the substrate-enzyme complex, in the hydrolysis of biosynthetic glucuronides by glucuronidase. Figures available for K_m are as follows: phenylglucuronide, 0.0035M (Kerr *et al.* 1948), bornylglucuronide, 0.01M, methylglucuronide, 0.004M, and oestriolglucuronide, 0.0005M (Fishman, 1939), phenolphthaleinglucuronide, 0.00005M (Talalay, Fishman & Huggins, 1946). Saccharic acid has a higher affinity for glucuronidase than all except one of these glucuronides. Changing the carboxyl at $\text{C}_{(6)}$ in saccharic acid to a primary alcohol group to give gluconic acid, or changing the configuration to give mucic acid, resulted in considerable diminution of the inhibitory power. The effect of glucuronic acid on the hydrolysis of phenylglucuronide by the enzyme may have been inhibition in the usual sense or a mass action effect. Hydrolysis of a glucuronide by glucuronidase is known to result in formation of free glucuronic acid (Levy, 1948).

The failure of saccharic acid in large doses to modify liver regeneration after damage, or growth in infant mice may indicate that the enzyme is not directly concerned in cell division, but the results are capable of explanation in other ways. Saccharic acid may be too rapidly metabolized or excreted to produce any perceptible changes *in vivo*. Alternatively, normal cell division may involve hydrolysis of a naturally occurring glucuronide with a much greater affinity for the enzyme than that of saccharic acid. Experiments designed to exclude the possibility that saccharic acid does not penetrate the intact cell were unsatisfactory on technical grounds, but the results, for what they were worth, suggested that penetration did occur. Preliminary results obtained by Dr J. G. Campbell (private communication) suggest that saccharic acid considerably retards hydrolysis of the glucuronide of '1 ortho-hydroxyphenylazo-2-naphthol' by frozen mouse kidney sections in the histochemical test of Friedenwald & Becker (1948).

As a result of the work of Levy *et al* (1948), it is no longer necessary to postulate a synthetic role for β glucuronidase in the body (Fishman, 1940) in order to explain the changes in the activity of the enzyme which can be produced in various organs. The view that β glucuronidase is not involved in glucuronide synthesis (Levy, 1948) receives support from the failure of saccharic acid to influence formation of *o*-aminophenylglucuronide by mouse-liver slices, and of β -glucuronidase preparations to effect condensation of glucuronic acid with *o* aminophenol.

Certain sex hormones are known to be excreted as glucuronides. The effect of administering saccharic acid on the metabolism of these compounds might repay investigation.

SUMMARY

1 Hydrolysis of phenylglucuronide by β -glucuronidase was strongly inhibited by saccharic acid. Closely related compounds were much less effective.

Inhibition by saccharic acid was competitive, and a value of $6 \times 10^{-5} M$ was obtained for K_i , the dissociation constant of the enzyme-inhibitor complex.

2 Saccharic acid had no marked effect on synthesis of *o*-aminophenylglucuronide by mouse-liver slices.

3 Administration of large doses of saccharic acid to mice did not influence liver regeneration after damage or growth in infant animals.

4 No conjugation of *o* aminophenol with glucuronic acid was observed after incubation in the presence of β glucuronidase.

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The Metabolism of Chrysene the Isolation of 3-Methoxychrysene by Methylation of the Phenolic Metabolite of Chrysene from Rat Faeces*

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Attention has been drawn to the fact that in mice and rats there is a remarkable similarity in the position of metabolic oxidation in the molecule for the three hydrocarbons 1,2-benzanthracene, 1,2,5,6-dibenzanthracene and 3,4-benzpyrene (Berenblum & Schoental, 1943). It was pointed out that these positions are not the ones which are chemically the most reactive. A preliminary report by Dickens (1945), that 9,10-dimethyl-1,2-benzanthracene is metabolized into its 4'-hydroxy derivative, brings

* A preliminary report on this work was communicated to the Biochemical Society (*Biochem J* 1945, 39, LXIV).

this hydrocarbon into line with those mentioned above, and provides additional support for the similarity in metabolic pattern.

It was thought that useful information might be derived from a study of the metabolism of chrysene, in which the anthracenoid ring structure is lacking. The following investigation was, therefore, undertaken.

EXPERIMENTAL

The procedure adopted was essentially the same as that used by the authors in their previous metabolic studies. Thirty rats were injected intraperitoneally with 2 ml of a warm,

supersaturated solution of purified chrysene (m p 251–252°) in arachis oil, representing about 25 mg /rat. The animals were kept in metabolism cages permitting the separate collection of urine and faeces. Their diet consisted mainly of rat cakes (North Eastern Agricultural Co operative Society Ltd, Aberdeen), supplemented occasionally with wholemeal bread and cod-liver oil, and water *ad lib*.

Isolation The faeces were collected daily, dried in air, ground to a fine powder, and extracted with benzene by percolation at room temperature. The benzene extracts were shaken with dry 'precipitated pure silica' (British Drug Houses Ltd.) in order to remove a fluorescent, strongly adsorbable material (? a conjugated metabolite), and the solution, after filtration, was passed through columns of alumina for chromatographic separation. On developing the columns with benzene, three main zones could be distinguished.

A (at the top of the column) A strongly adsorbed zone, greyish in ordinary light, with a weak violet fluorescence in ultraviolet light. (This contained some of the fluorescent material mentioned above, most of which had been adsorbed on the silica.)

B (appearing below *A*) A narrow brownish zone, with a bluish fluorescence in ultraviolet light. (This contained the phenolic metabolite.)

C (appearing below *B*) A wide zone exhibiting a weak greenish fluorescence in ultraviolet light. (This zone contained the bulk of the sterols present in the extract.)

Zone *B* was cut and extracted with methanol. The extract was concentrated under reduced pressure, methylated by treatment with dimethyl sulphate and sodium methoxide in the cold, and extracted with benzene in the usual manner. The methylated product was purified by repeated chromatographic adsorption from light-petroleum solution, the fluorescent zone being extracted by benzene after cutting up the column each time. On concentration, the final eluate deposited a crystalline substance. This was recrystallized from light petroleum, and yielded colourless, fluorescent leaflets, m p 185–186°. It formed a complex with 2,7-dinitro anthraquinone in xylene, which crystallized in fine crimson needles, m p 282–284°.

Identification For reference, the six isomeric monomethoxychrysenes were synthesized, and their 2,7-dinitro anthraquinone complexes prepared, by one of us (R. S.) in collaboration with Prof. J. W. Cook, F.R.S. (Cook & Schoental, 1945).

The properties of the methylated metabolite were found to correspond closely to those of 3-methoxychrysene as regards the colour and crystal form of the compounds and their respective complexes, the melting points of the complexes agreed closely, but the value for the methylated metabolite itself (185–186°) was higher than that quoted previously (Cook & Schoental, 1945) for 3-methoxychrysene (167–168°). Therefore, a synthetic specimen of 3-methoxychrysene was further purified (at the suggestion of Prof. Cook) by regeneration from the complex with 2,7-dinitro anthraquinone by chromatography, and it had a melting point of 185–186°. The identity of the methylated metabolite with 3-methoxychrysene was confirmed by lack of depression of the melting points of mixtures of the two compounds, as well as of their dinitroanthraquinone complexes.

In our earlier metabolic studies on 3,4-benzpyrene (Berenblum, Crowfoot, Holiday & Schoental, 1943) and 1,2-benzanthracene (Berenblum & Schoental, 1943), the amounts of metabolite were often so small as to preclude

the possibility of obtaining them in pure enough form for melting point determinations, and reliance had to be placed on fluorescence and ultraviolet absorption spectrography, and on other micromethods of identification. In the present investigation these methods also proved useful, especially in the early stages, before the methylated metabolite was available in crystalline form, when comparison of its fluorescence spectrum with those of the synthetic methoxychrysenes gave an early indication of the position of substitution.

Table 1 *Positions of fluorescence spectral bands of chrysene, methylated metabolite of chrysene, and the six isomeric monomethoxychrysenes*

	Wavelengths of main bands (m μ)
Chrysene	(366), 382, 404
Methylated metabolite	368, 386, 408
3-Methoxychrysene	368, 386, 408
4-Methoxychrysene	369, 387, 410
2-Methoxychrysene	369, 389, 412
6-Methoxychrysene	371, 389, 412
5-Methoxychrysene	373, 391, 415
1-Methoxychrysene	374, 393, 418

For greater accuracy of measurement of the fluorescence bands, a method was employed for obtaining 'peak' photographs (in place of the semi-quantitative representations used in our previous publications), by incorporating a 'moving wedge' behind the slit of the spectrograph. Details of the method, and reproductions of the fluorescence spectra of the six isomeric methoxychrysenes are given elsewhere (Berenblum & Schoental, 1946a). In Table 1 are given the positions of the main bands. The distinct differences between the spectra of the different isomers gave an indication of the relative specificity of fluorescence spectrography for this class of compounds. The fact that the spectrum of the methylated metabolite was indistinguishable from that of 3-methoxychrysene confirmed their identity.

A study of the ultraviolet absorption spectra of this series of compounds was undertaken by Holiday & Jope (unpublished results). They found very distinct differences in the absorption spectra of the six isomeric methoxychrysenes, and reported that the spectrum of the methylated metabolite was identical with that of 3-methoxychrysene, thus providing further confirmation of their identity.

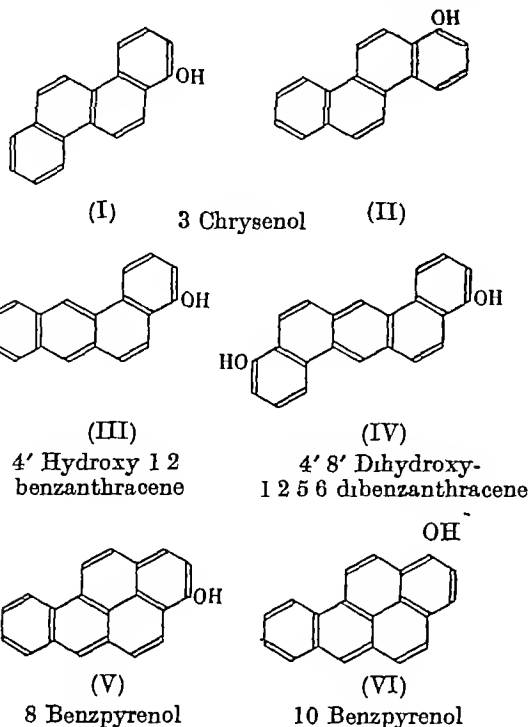
DISCUSSION

Since the methylated metabolite of chrysene has now been identified as 3-methoxychrysene, and since the metabolite itself possesses the properties of a phenol, it may be concluded that the chrysene metabolite present in the rat faeces is 3-chrysenol.

This result is in keeping with the principle, previously noted in connexion with other polycyclic hydrocarbons (Boyland & Levi, 1935; Berenblum & Schoental, 1943) that metabolic oxidation does not occur in the positions of the molecule which are chemically the most reactive, i.e. the 2-position in the case of chrysene (Newman & Cathcart, 1940).

In comparing the structural formula of the rat metabolite of chrysene (I or II) with those of 1,2-benzanthracene (III), 1,2,5,6-dibenzanthracene

(IV), and 3 4-benzpyrene (V and VI), a similarity in pattern of metabolic oxidation is clearly discernible. It would seem, at first sight, that this



similarity is dependent on the manner of presentation, i.e. that formula I corresponds to 8-benzpyrenol, and formula II to 10 benzpyrenol. In fact, the feature common to them all is that the sites of metabolic oxidation are all in the alpha position to the phenanthrenoid 9 10 double bonds, and, since benzpyrene has two such double bonds (1 2 and

6 7), there are two corresponding alpha positions of metabolic oxidation (Berenblum & Schoental, 1946b).

An indication of the chemical mechanism of metabolic oxidation is given by the finding by Boyland & Levi (1935) that animals fed on anthracene excrete 1 2 dihydroxydihydroanthracene, and that this product readily undergoes conversion into 1-anthranol by mild acid treatment. Similar conversions of hydrocarbons to diols in the animal body have been observed in the case of naphthalene and phenanthrene (Young, 1947, Booth & Boyland, 1947, Boyland & Woolf, 1948). It thus seems a plausible hypothesis (Fieser, 1941) that a dihydroxydihydro derivative might constitute an intermediate stage in the metabolic conversion of other hydrocarbons into their respective phenols.

The results with chrysene, described in the present communication, are not inconsistent with this view. However, this would not explain why the supposed 'perhydroxylation' does not take place in the reactive, adjacent, 1- and 2-positions (which so readily yield to oxidation, see Bamberger & Burgdorf, 1890), unless these positions are already blocked by some group, possibly enzymes, in the process of metabolism.

SUMMARY

After intraperitoneal injection of chrysene into rats, a phenolic fraction was isolated from their faeces. The isolated product yielded on methylation a compound identified as 3-methoxychrysene, indicating that the phenolic metabolite was 3-chrysenol.

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Bile Acid Enteroliths; with an Account of a Recent Case

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Enteroliths consisting mainly of choleic acid have been reported on at least ten previous occasions, the first by Morner & Sjoqvist (1908, quoted by Morner, 1923) Morner (1939) gives a list of cases known to him up to that time The only case reported from this country is the one described by Raper (1921), the remainder having been found in Sweden Chitre & Puradare (1944) described an enterolith weighing 185.5 g removed from a Hindu woman, which was said to contain over 70 % of glycocholic acid, but no details of the analysis nor physical constants of the bile acid were given

On 21 March 1948, Mr G Armitage, Honorary Surgeon to the Leeds General Infirmary, removed two stones from the intestine of a woman aged 73, and submitted them to me for analysis One of the stones was found in a duodenal diverticulum, and the other lower down the small intestine, it was thought that this too had been formed in a diverticulum, but later escaped from it and gave rise to the symptoms of intestinal obstruction which were the outstanding clinical features of the case A more complete account of the clinical and radiological aspects of the case will be published elsewhere (Armitage, Fowweather & Johnstone, 1949)

The stones were yellowish brown, of irregular shape, with a roughness similar to that of the skin of an orange The smaller stone weighed 26 g with an overall length of 41 mm and overall width of 21 mm On sawing into halves, it was seen that there was a central zone, rectangular in section, measuring 10 × 7 mm of a much darker colour than the rest, the demarcation line between central and outer zones was not very sharp The larger stone weighed 29.5 g, had overall dimensions of 47 and 45 mm and a darker central zone, oval in section, measuring 30 × 20 mm

EXPERIMENTAL

Analysis was begun on the outer zone of the smaller stone, the inner zone appeared to differ from it only quantitatively Later the larger stone was examined, the whole of one half was ground up and analyzed, and the central zone of the other half analyzed separately At the centre of the larger stone was a thin irregular piece of vegetable material measuring 20 × 5 mm, thought to be a portion of a fruit skin, nothing of this kind was found within the smaller stone

Cholesterol was tested for by the Liebermann Burchard reaction, which was found to be negative The Pettenkofer reaction, however, was strongly positive The material con-

tained only a very small amount of mineral matter, was soluble in hot ethanol, and readily soluble in alkali, giving a solution which frothed readily, the froth persisting for some time It did not melt at 100° It was evident that the stone consisted largely of bile acid

Lipids were removed from a weighed quantity of dry, powdered material by extraction in a Soxhlet with light petroleum The residue was extracted with ethanol and the extract evaporated to dryness

RESULTS

The following figures were obtained

	A Smaller stone, outer zone	B Larger stone, whole of one half	C Larger stone, inner zone
Water	1.80	0.56	0.20
Ash	0.40	0.34	0.30
Free fatty acids	1.85	2.04	13.29
Neutral fat	0.30	0.44	4.91
Ethanol extract (mainly bile acid)	92.20	93.71	74.05
Food residues, etc (by difference)	3.45	2.91	7.25
	100.00	100.00	100.00

The ethanol extract contained a small amount of bile pigment

The dried ethanol extracts were redissolved in ethanol and titrated with 0.1 N-NaOH, using phenolphthalein as indicator, showing the equivalent weights to be A, 379.5, B, 382.8, C, 386.2 A further portion of the petroleum extracted material was dissolved in ethanol, treated with animal charcoal and filtered The filtrate was evaporated to small bulk, from which most of the dissolved material crystallized out The crystals were filtered off, washed with ethanol and dried in a vacuum desiccator The resulting product was entirely colourless and had the following characteristics

	A	B	C
Melting point	185–186°	185–186°	186–187°
Equiv wt	378.8	377.4	382.1
$[\alpha]_D^{20}$	+48.7°	+47.5°	Not determined

Wieland & Sorge (1916) gave the melting point of choleic acid as 186–188°, and its optical rotation as +48.47° Raper (1921) found for the recrystallized ethanol extract from his stone a melting point of 186.5–187.5° and optical rotation of +50.52°, the latter figure is higher than most other recorded figures, he stated that the molecular weight of his

product, determined by titration with 0.1 N NaOH, was 384.9. The choleic acids normally encountered are complexes containing eight molecules of deoxycholic acid and one molecule of a higher fatty acid. Wieland & Sorge (1916) consider that natural choleic acid contains both palmitic and stearic acids. The equivalent weights of the palmitic and stearic complexes are 376.9 and 380 respectively.

The similarity in appearance and behaviour, as well as in physical constants, of the ethanol extracts from the materials *A*, *B* and *C* indicated a close similarity in composition. Moreover, the evidence obtained indicated that the extracts consisted largely of natural choleic acid, and the crystalline products obtained from them showed a high degree of purity. This was confirmed by a determination of the deoxycholic acid content of one of them by the method of Kawaguchi (1938). It showed a value of 92.3%, while the average figure for the deoxycholic acid content of natural choleic acid obtained by Wieland & Sorge (1916) was 91.8%.

In the cases already reported, it has been shown that choleic acid was the principal constituent of the ethanol extract of the stones examined, but little or no information has been given as to the nature and quantity of other substances present. In the present case attempts were made to obtain more precise information on this point. Bilirubin was certainly present in small amount, sufficient to give a definite yellow colour to the ethanol extracts. That some other material was also present was indicated by the fact that the mother liquor and washings after separation of the crystalline products from decolorized extracts of *A*, *B* and *C*, when added together and concentrated, yielded a crystalline product of melting point 180–182°, i.e. about 5° below the melting point of pure choleic acid. Experiments were therefore made on the whole of the ethanol extract (Material *B* was used for the purpose). When submitted to the Gregory & Pascoe (1929) reaction the extract gave a definitely positive result. This reaction, especially in the more recent modifications of Reinhold & Wilson (1932) and Irvin, Johnston & Kopala (1944) has been shown to be highly specific for cholic acid. Using the last named method the amount of cholic acid in the whole ethanol extract was found to be approximately 10%. An almost identical result was given by the method of Kawaguchi.

The molecular weight of cholic acid ($C_{24}H_{40}O_5$) is 408. Hence admixture of cholic acid with choleic acid will cause the equivalent weight to be higher than for pure choleic acid, and the equivalent weights of the total extracts have, in fact, been shown to be higher than for the crystallized products, a mixture containing 90% of choleic acid and 10% of cholic acid would have an equivalent weight of 382. It is unlikely that the conjugated cholic acids, glycocholic

and taurocholic acids, are present to any appreciable extent, for their molecular weights are 465 and 515 respectively. Even small amounts would therefore have a considerable effect on the equivalent weights.

It may reasonably be accepted, therefore, that the total ethanol extract of the stones consisted of bile acid together with a small amount of bile pigment, and that the bile acid contained approximately 90% of choleic acid and 10% of cholic acid, the conjugated bile acids were not present to any appreciable extent.

In bile much the greater proportion of the bile acids is present in the conjugated form, and as salts (mostly sodium), while in the enteroliths they are present as free acids, and unconjugated. An experiment to determine the pH at which certain bile acids are precipitated from solutions of their sodium salts gave the following results:

Bile acid	pH at which precipitation occurred
Choleic acid	7.0
Cholic acid	5.8–6.0
Glycocholic acid	4.6–4.8

Josephson (1933) performed somewhat similar experiments with a number of bile acids with similar results. He found the dissociation constants of deoxycholic acid, cholic acid and glycocholic acid to be 3.8×10^{-7} , 6.4×10^{-7} and 355×10^{-7} respectively, for the tauro acids the constants are higher than for the glyco acids.

DISCUSSION

True enteroliths of any kind are rare. The ease with which many foreign bodies pass through the normal alimentary canal makes it obvious that for enteroliths to form and grow in size some mechanical condition must be present which allows the retention of concretions within the intestine in their early stages. In almost all the reported cases such a mechanical condition was noted, in some the stones were obviously related to the presence of diverticula, in a few to stricture. The presence of foreign bodies is also likely to be favourable to stone formation since their presence will cause stasis (an important factor in stone formation) either in a diverticulum or in the lumen of the intestine, and they will also act as a nucleus around which solid material may be deposited. Helstrom (1936) found a plum stone at the centre of a choleic acid enterolith. Raper (1921) found tissue resembling fruit skin, similar material was found in the larger of the two stones in the present case, and Chitre & Puradare (1944) found a tamarind seed.

There must be in addition special conditions which determine the formation of bile acid stones. With one exception the reported cases already referred to have been choleic acid stones, and the exception, that of Chitre & Puradare (1944), is not sufficiently well

documented to place the identification of the bile acid concerned entirely beyond doubt. This finding at first seems surprising since the conjugated bile acids are normally present in much greater concentration than choleic acid. McGee & Hastings (1942), in experiments on human subjects, found that the pH of the upper part of the small intestine was between 6.2 and 6.8 except in a few isolated instances. The experiment already described shows that choleic acid is the only one of those examined which will be precipitated within this range, and thus offers a convincing explanation why choleic acid is precipitated in preference to other bile acids. It is probable that in some cases the acidity in this region exceeds the normal (e.g. where the amount of hydrochloric acid discharged from the stomach into the duodenum is greater than usual as in conditions associated with hyperchlorhydria), and may reach a degree of acidity sufficient to cause precipitation of cholic acid also, but the attainment of an acidity sufficient to precipitate glyco or tauro acids is very much less likely.

The formation of stones within the intestine would appear to require the precipitation of the material concerned in appreciable quantity, yet it is believed that unconjugated bile acids are present in bile in quite small amounts. Doubilet (1936) gives some results of 'typical examples' of differential bile acid analysis, these show, in human gall-bladder bile, 0.890% of unconjugated acids, out of a total of 9.751%, and in human fistula bile 0.413% out of a total of 1.717%. Not all of the unconjugated acids is deoxycholic acid, capable of combination with the free fatty acid in bile or in the intestine to form choleic acid. I have not seen any separate figures for unconjugated cholic and deoxycholic acid in bile, but it is generally believed that the total cholic acid exceeds the total deoxycholic acid. Wieland & Revery (1924), who noted the presence of chenodeoxycholic acid in human bile, gave the proportions of cholic, deoxycholic, chenodeoxycholic acid as 3:1:1. Doubilet's (1936) figures for human gall-bladder bile show 4.255% of cholic acid and 5.496% of deoxycholic acid, he makes no mention of chenodeoxycholic acid, but, if any were present, it would be included in the figure for deoxycholic acid. (Here it may be noted that Nagaki & Ohshima (quoted by Saba, 1940) claim that chenodeoxycholic acid is often found to predominate in human bile.) Figures for deoxycholic acid therefore, in cases in which examination for, or determination of, chenodeoxycholic acid is not made, may well be too high. Chenodeoxycholic acid, like cholic acid, has little tendency to form co-ordination compounds (Sobotka, 1937a), hence its presence will not result in the formation of choleic acid. If, then, the proportion of the unconjugated deoxycholic acid in bile bears any relation to that of the total deoxycholic acid, the

amount of the former (and therefore of choleic acid) is normally very small, it is in fact so small as to indicate that deposition and retention within the intestine, in quantities sufficient to form the enteroliths which have been described, is unlikely to occur. Raper (1921) suggested that the material first deposited was glycocholic acid and that glycine was subsequently split off from this by bacterial action. There are, however, three difficulties in accepting this explanation. First, there is not, according to Sobotka, any real evidence that the conjugated bile acids form choleic acids. Secondly, the evidence for cleavage of conjugation in the manner suggested is conflicting (for references see Sobotka, 1937a), and lastly the pH required for the deposition of the free conjugated acids is definitely lower than that found in the intestine.

There are, however, other and more likely explanations why in some cases the choleic acid of the bile may be higher than is suggested by the figures already quoted. In the first place, the number of published differential bile acid analyses of human bile is small, and they give no indication of the variations which might occur normally in the amount and proportion of the different acids, in this connexion it has also to be remembered that the methods of analysis available for this purpose leave much to be desired. In the second place, there is some evidence that the proportion of conjugated to unconjugated bile acid, and of cholic acid to deoxycholic acid, etc., may be altered by disease. The liver is presumably the site of conjugation, and an increase in unconjugated bile acid has been noted in disease of that organ. (For references see Sobotka, 1937b.) An increase in the proportion of deoxycholic acid to cholic acid has also been noted, mainly in pathological conditions, but Sobotka thinks this is more likely to be due to a decrease in production of cholic acid than an increase of deoxycholic acid. Disease of the gall bladder, by altering its capacity to absorb various substances from bile, may also alter the bile acid composition of this fluid.

It will be seen, therefore, that the formation of choleic acid stones requires the concurrence of a number of factors, viz. a mechanical condition capable of allowing the retention of precipitated acid, bile which contains more deoxycholic acid than is usually present, and, also, bile which contains a considerable proportion of deoxycholic acid in the unconjugated form, whether these bile changes represent the extreme of normal variation, or are the result of disease cannot be decided on present knowledge of the bile acid composition of bile. Hyperchlorhydria may be an additional factor. The presence of retained indigestible matter in the intestine is also of some importance.

One other fact has not yet been mentioned, namely that all the reported cases have occurred in women.

Why this should be so is not clear, and can only be a matter for speculation. The fact that the production of choleic acid enteroliths requires the concurrence of a number of factors explains their rarity. Nevertheless, certain considerations suggest that they may not be so rare as the small number of reported cases would indicate. Of the ten previously reported cases, five have been recorded by one man (Mörner, 1939) and three by another (Helstrom, 1936). That one keen observer should have identified half the known cases does seem to show that others must have been missed, there is in fact much evidence in the literature that some enteroliths have not been examined at all, while some have received insufficient examination to identify their constituents with certainty. Any real knowledge of the incidence of bile acid enteroliths—or indeed of any type of enterolith—can only be obtained if all stones found in the intestine are submitted to careful chemical analysis.

SUMMARY

1 Two enteroliths removed from the intestine of a woman have been analyzed, and it has been shown that they contained over 90% of bile acids. Of these

acids approximately 90% was choleic acid, and 10% was cholic acid.

2 The pH at which certain bile acids are precipitated from solutions of their salts has been determined, and it has been shown that, of the bile acids usually present, choleic acid is much the most likely one to be precipitated at the reaction normally found at the site of formation of these enteroliths. This is believed to explain why all the bile acid enteroliths so far reported, except one, consist mainly of choleic acid.

3 The factors which might be responsible for the formation of choleic acid enteroliths have been discussed and some deficiencies in our knowledge of the bile acid composition of human bile have been pointed out.

4 While bile acid enteroliths are undoubtedly of rare occurrence, it is considered that they are probably commoner than the number of reported cases indicates. This is believed to be due to insufficient examination of intestinal stones in some cases, and lack of any examination in others. Careful chemical examination of all intestinal stones is recommended.

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The Bence-Jones Protein of Multiple Myelomatosis. its Methionine Content and its Possible Significance in Relation to the Aetiology of the Disease

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Few chemical investigations have been made on Bence-Jones protein in recent years. Reports on the methionine content of the protein are particularly scanty since, at the time of the earlier work (Hopkins & Savory, 1911, Abderhalden & Rostoski, 1905), this amino-acid was unknown. The values reported for methionine (Calvery & Freyberg, 1935, Devine, 1941, Harvier & Rangier, 1943) show considerable

variation, but agree upon a low figure. As a patient with multiple myelomatosis, who was excreting large quantities of Bence Jones protein, had come to our notice, it was decided to investigate again the content of methionine and other amino acids. The quantities excreted by the patient permitted purity tests on the intact protein, and the use of five independent methods for determining the methionine content

It was also hoped that the material might provide a source of methionine-free protein in rat feeding experiments designed to produce anaemia and hepatic necrosis

Case summary

The patient, a man aged 44, was admitted to University College Hospital, under Prof H P Himsworth on 3 August 1946, complaining of weakness, loss of weight and pain in the lower ribs on the left side. On examination he appeared pale and the pain in the ribs was found to be accentuated by direct pressure. The urine on warming gave a heavy curdy precipitate at about 60°, which completely redissolved when it was heated above 80°, and reprecipitated on cooling below 80°. No other abnormal constituents were found. A further examination of the urine for Bence-Jones protein was made with the tests described by Osgood & Haskins (1931). Typical reactions were given with salt and acetic acid as well as with sulphosalicylic acid. Investigations during the next few days showed blood urea 36 mg/100 ml, plasma proteins 6.2 g/100 ml, albumin 4.7 g/100 ml, albumin/globulin ratio = 3.1, haemoglobin 66%, white cells 6700/cu mm with normal differential. Sternal marrow examination showed that 55% of the nucleated cells were plasma cells. Radiography of the skeleton showed a few clearly defined decalcified areas in the skull and vertebrae consistent with multiple myeloma deposits. Similar areas were much more widespread in the lower ribs, humeri, femora, pelvis and clavicles. The patient was put on a high protein (200 g daily) diet and treated with nitrogen mustard (methyl bis (β chloroethyl)amine hydrochloride). His anaemia worsened, however, and the haemoglobin fell to 42% in about 6 weeks, when he was given blood transfusions and allowed to go home.

He was admitted again on 9 December 1946 with further loss of weight, lassitude and pain in the ribs. He was beginning to become breathless on exertion. His blood urea was now 78 mg/100 ml, haemoglobin 32%, red cell count 1.6×10^6 /cu mm, white cell count 6000/cu mm, of which 2% were myelocytes and 1% plasma cells. Plasma proteins were 6.2 g/100 ml, albumin 4.5 g/100 ml, albumin/globulin ratio 2.7, sedimentation rate 65 mm in 1 hr. He was treated by means of blood transfusions and given a course of methionine, 5 g by mouth daily for 10 days. His condition continued to get worse, he began to be irrational, and, when very ill, insisted on going home where he soon died.

Unfortunately, the plasma proteins were not examined by electrophoresis.

METHODS

Collection of urines. Urine was collected over periods of 24 hr and stored at 5°, and preserved by thymol.

Determination of protein content. The volumes of 24 hr collections of urine were measured. The urine was filtered, and 5 ml introduced into a centrifuge tube. It was made just acid to litmus with acetic acid, and heated on a water bath to 70°. The coagulated precipitate of protein was spun down, washed once with distilled water, twice with 90% ethanol and once with absolute ethanol. Finally the precipitate was dried in the oven at 100° for 2 hr, weighed and rechecked to constant weight.

Preparation of large sample of protein. Urine was treated, in 7 l amounts, with 90 g of active charcoal for 15 min, and filtered through fluted filter paper. The clear, pale yellow

liquid, made just acid to litmus with acetic acid, was heated with mechanical stirring to 55–65° to produce precipitation and coagulation of the protein. The precipitate was filtered and washed with distilled water until chloride free, and spread out on Petri dishes, which were placed on top of an oven at 90° overnight. The dried protein was put through a mincer and gave a white powder, quite tasteless and odourless. The yield was about 95 g/7 l urine. The protein contained no phosphorus.

By this procedure, a total of 1350 g of protein was collected and thoroughly mixed. This denatured protein was later used for the feeding experiments and analyses.

Analysis of protein by salting out methods. The filtered urine in volumes of 280 ml was dialyzed against running tap water for 48 hr, and against a large volume of static distilled water for a further 48 hr. It was filtered from the slight precipitate and the filtrate used for salting out experiments.

A constant volume of the dialyzed protein solution was treated with varying quantities of saturated Na_2SO_4 solution and distilled water to give a series of solutions having a volume of 10 ml, a nitrogen content of 10.5 mg, a pH of 7.0, and with the Na_2SO_4 concentrations ranging from 30 to 50% saturation at 37°, at which temperature the whole subsequent procedure was also carried out. Having stood for 22 hr, the mixtures were filtered, and the filtrates analyzed for nitrogen.

To isolate the first fraction of protein precipitated by this method, 1700 ml of urine were dialyzed by the above method, and the protein solution treated with Na_2SO_4 to bring to 38% saturation. Under these conditions 29% of the nitrogen was precipitated. The mixture was filtered, and the precipitate taken up in distilled water and dialyzed as above to give fraction A.

To investigate further the upper part of the salting-out curve, 10 ml fraction A, containing 10.0 mg N, were treated with varying quantities of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ at 37°. The protein left in solution was estimated as in the salting out experiment.

Electrophoretic analysis of protein. Urine (250 ml) was freeze dried and sent to Dr A L Alling of the Strong Memorial Hospital, Rochester, N Y, U S A, to whom we are indebted for the electrophoretic study. After suitable dialysis of the urine against sodium diethylbarbiturate buffer of pH 8.5 and ionic strength 0.1 the electrophoresis was carried out for 3 hr in the tall form of the standard 11 ml Tiselius cell at a field strength of 6.8 V/cm. Boundary patterns were obtained by the Longworth scanning method (Longworth, 1939).

Chromatographic analysis for amino acids. The denatured protein was hydrolyzed for 24 hr at 100° with 6N-HCl, and, after removal of the HCl by distillation *in vacuo*, the residue was dissolved in water. An amount of the solution containing 50 μg of N was analyzed for amino acids on the two-dimensional phenol 'collidine' paper chromatogram of Consden, Gordon & Martin (1944). A further similar quantity, previously treated with 10 μl of 30% (w/v) H_2O_2 , was analyzed similarly for the specific purpose of revealing methionine and cystine if present (Dent, 1947). These two analyses were later repeated with 10 times the above amounts of hydrolysate with the object of detecting constituents present in small quantities only. One dimensional chromatograms were also carried out in collidine, for subsequent spraying with the Pauly reagent (Pauly, 1904) for detection of histidine and tyrosine, and in *tert* amyl alcohol (Work, 1948) to detect the two leucines, which overlap in the other solvents.

Analyses for cystine (other than by paper chromatography)
Qualitative To test qualitatively for cystine in a protein hydrolysate, the nitroprusside test may be inconvenient on account of the colour of the hydrolysate itself, and the quantity of protein required. The following spot test was used.

A drop of solution which may contain about 0.1% (w/v) cystine is applied to a filter paper, which is allowed to dry, immersed in 5% $\text{Hg}(\text{NO}_3)_2$ solution, thoroughly washed with distilled water and dried at room temperature or in an oven at 100°. The same concentration $\text{Hg}(\text{NO}_3)_2$ is striped across the paper from a micropipette, and is followed by 0.112N-KI applied in the same way. The background appears red on account of HgI_2 formation, whilst the area of cystine impregnation appears colourless for low concentration of cystine, and pale yellow for higher concentrations.

The following substances give a positive reaction to the test: cystine, cysteine, glutathione, homocysteine, cystine disulphoxide, thiolhistidine, *S* benzylcysteine. The following substances give negative results: *S* methylcysteine, taurine, cystathionine, methionine sulphone, methionine sulfoxide, lanthionine, $\beta\beta\beta'\beta'$ -tetramethylcystine, ergothionine, homocysteine thiolactone, glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, glutamic acid, arginine, lysine, histidine, phenylalanine, tyrosine, tryptophan, proline and methionine.

Cystine, however, can be distinguished from all other substances which give positive reactions by repeating the test, this time omitting the preliminary immersion in $\text{Hg}(\text{NO}_3)_2$ and subsequent washing and drying. Under these conditions cystine gives a negative result, but the other substances continue to give the same reaction as before.

The test will detect 2 μg of cystine with ease.

Quantitative Cystine and inorganic sulphur were determined gravimetrically by the method of Lugg (1938).

Analyses for methionine (other than by paper chromatography)
Chemical The nitroprusside colorimetric method of McCarthy & Sullivan as modified by Horn, Jones & Blum (1946) was used. The method is not, however, very accurate for low concentrations of methionine in protein hydrolysates. Methionine was also determined by subtracting cystine and inorganic sulphur (as determined above) from total sulphur as determined gravimetrically by a wet-ashing method (Masters, 1939).

Microbiological This was carried out by Mr J. G. Heath at the Cereals Research Station, St Albans, by the method of Dunn, Shankman, Camien, Frankl & Rockland (1944) as modified by Barton Wright (1946) with the organism *Leuconostoc mesenteroides* P60.

Biological Two animal feeding experiments were carried out. In both cases white Wistar rats were housed separately in metabolism cages, and a constant intake of dietary protein N (123 mg/rat/day) was provided, the carbohydrate supplied being appropriately reduced when the appetite was poor, so that minimal amounts of food were left uneaten. The cages, donations of carbohydrate, salt, arachis oil, cod liver oil, yeast extract, aneurin, riboflavin, pyridoxine, calcium pantothenate, choline and tocopherol were exactly as described by Dent & Ramington (1947), except where otherwise stated.

In the first experiment, two male rats of about 100 g were fed with casein as the sole source of protein N. After 20 days the casein was replaced by Bence Jones protein. After a further 14 days, 19 mg α aminobutyric acid/rat/day were included in the diet, as part of another experiment not

reported here. After a further 14 days, instead of this supplement, 27 mg DL-methionine/rat/day were given.

In the second experiment, three male and three female rats, all litter mates, and weighing about 115 g if males and about 93 g if females, were fed with casein for the first 14 days as above, except that the carbohydrate was mixed with CaHPO_4 in the proportion of 99.2% corn starch and 0.8% CaHPO_4 . The salts, apart from the CaHPO_4 , were supplied by adding a solution of the composition of the salt mixture of Osborne & Mendel (1919), except that the metallic sulphates were replaced by the equimolecular amounts of their chlorides. In this way an adequate salt mixture completely free of sulphur was provided.

For the rest of the experiment rats 1 and 2 remained on the same diet, to serve as controls, while all the other rats were given Bence-Jones protein instead of casein. After the first 14 days, however, rats 3 and 4 received the Bence-Jones protein alone, while rats 5 and 6 received the same protein and in addition 27 mg DL-methionine/rat/day. After a further 18 days the methionine supplement was switched from rats 5 and 6 to rats 3 and 4.

Throughout both experiments, 16 mg L-tryptophan/rat/day were added to the casein diet. Water, distilled in the second experiment, was supplied *ad lib*. The rats were weighed individually at least every other day.

Other analyses *Ash* A small quantity of protein was ignited at red heat for 45 min and the residue weighed.

Total nitrogen This was determined by the micro Kjeldahl method, with CuSO_4 and SeO_2 as catalysts.

Carbohydrate This was determined by the orcinol method of Ramington (1940).

RESULTS

Daily output of Bence Jones protein The output varied by about ± 10 g/day (Fig. 1). On normal hospital diet, with a protein intake of about 100 g,

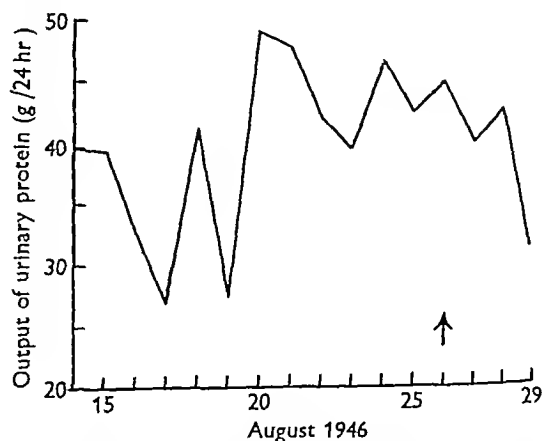


Fig. 1 Patient's excretion of Bence-Jones protein from 14 to 29 August 1946, representative of the 62 days on which it was determined at various stages of the illness. On 26 August (indicated by an arrow) he was given by mouth 50 g of his own Bence-Jones protein in addition to his normal diet.

the daily Bence-Jones protein output averaged 34.5 g over a 5 day period. For the next 9 days, on a diet containing about 200 g protein, the daily

output averaged 38.4 g. The average output remained at about the same level throughout the period during which the patient was studied, and was 36 g when averaged over the 62 recorded days. After eating 50 g of his own recovered Bence Jones protein, in addition to his normal diet, the patient showed no change in the output. (A healthy volunteer also ingested 50 g of the protein, without producing proteinuria or other detectable effects.) After the patient had been transfused with 2 pints of blood the output was halved for 2 consecutive days, but after a second transfusion about 2 months later a similar result was not reproduced. The feeding of 50 g of DL methionine/day for 10 days did not affect the output, and methionine could not be detected in the daily urines by the one-dimensional chromatographic technique of Dent (1946) using phenol as the solvent. Since the method would detect 2.5 μ g of methionine, and the average urine volume was 2500 ml, the daily output of this amino-acid, while it was being given by mouth, must have been less than 250 mg. α -Aminobutyric acid was also not detected in the urine. Methionine is readily detected in this way in the urine of a normal person who ingests 5 g/day.

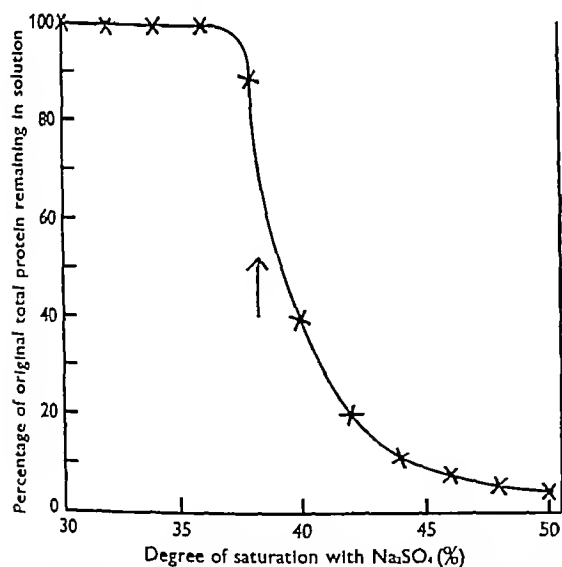


Fig 2 Salting out curve of Bence Jones protein by Na_2SO_4 at 37° . The arrow indicates the point at which the bulk fractionation was made (see text).

Salting out curves Fig 2 shows the effect of salting out the dialyzed Bence-Jones protein with sodium sulphate. To find the number of constituents, the log of the solubility of the protein was plotted against salt concentration (Fig 3, see also Cohn, 1925). The precipitation graph then follows along two straight lines, representing two constituents, with the point of inflexion when 87.8% of the nitrogen has been precipitated. The arrows in Figs 2 and 3 indicate the point at which the fractionation described on p 611 was made. The salting-out curve of

this fraction (fraction A) with zinc sulphate is shown in Fig 4.

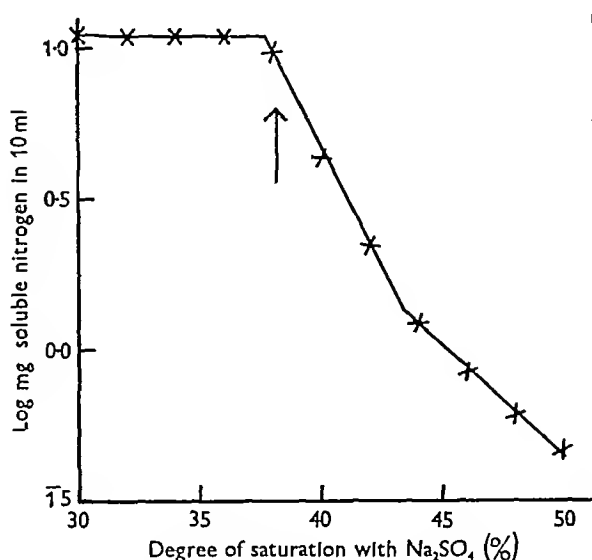


Fig 3 The results of Fig 2 plotted on the log scale to show the number of components present. The arrow indicates the point on the curve above which it was more carefully examined in another experiment (see text and Fig 4).

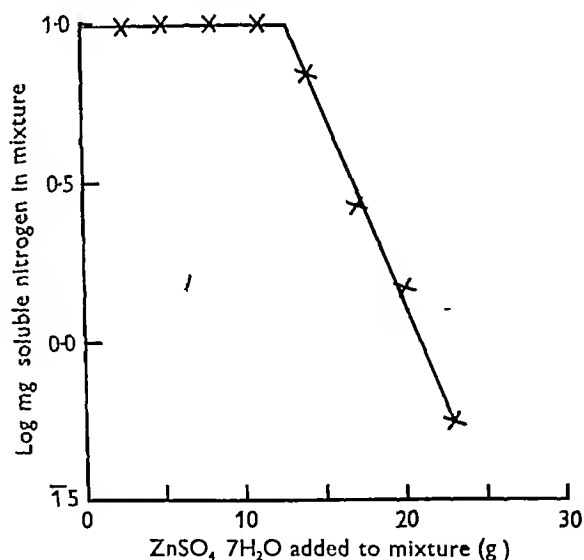


Fig 4 Salting out curve of fraction A of Bence-Jones protein by ZnSO_4 at 37° .

Electrophoretic diagrams Dr Alling reported 'The electrophoretic mobility of different samples of Bence Jones proteins varies somewhat, but the mobility in this case corresponds to that which we have found most frequently for Bence-Jones protein. The pictures (Fig 5) show the usual boundary anomalies and one large slightly asymmetrical peak moving with the mobility of fibrinogen. The asymmetry of the main peak probably represents a small amount of protein travelling slightly faster than the main component.'

When this undenatured protein was added to a sample of normal plasma, the peak migrated with the fibrinogen

Analysis by paper chromatography The results of one of the two dimensional chromatograms are shown diagrammatically in Fig 6 It shows all the

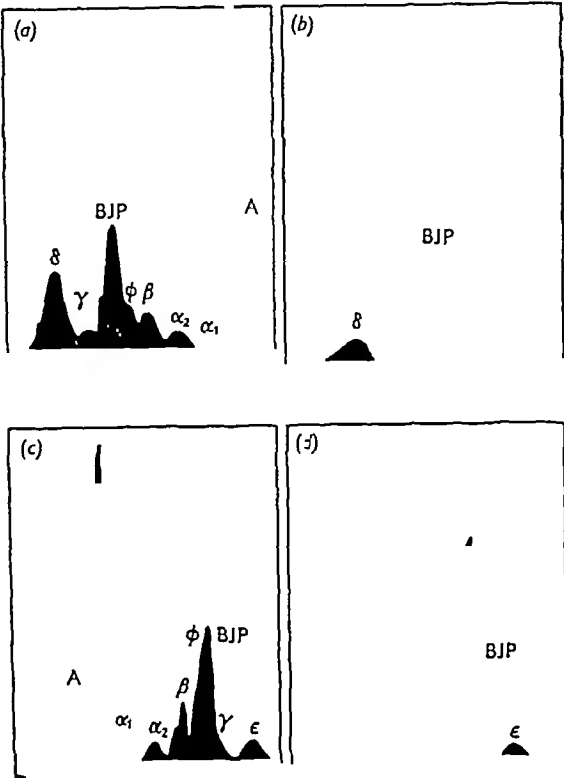


Fig 5 Electrophoretic patterns of Bence-Jones protein (a) and (b) are the ascending, (c) and (d) the descending patterns In (a) and (c) the protein has been mixed with plasma proteins from a normal man A=albumin α_1 , α_2 , β and γ =the corresponding globulins ϕ =fibrinogen δ and ϵ are the usual boundary anomalies BJP=Bence Jones protein

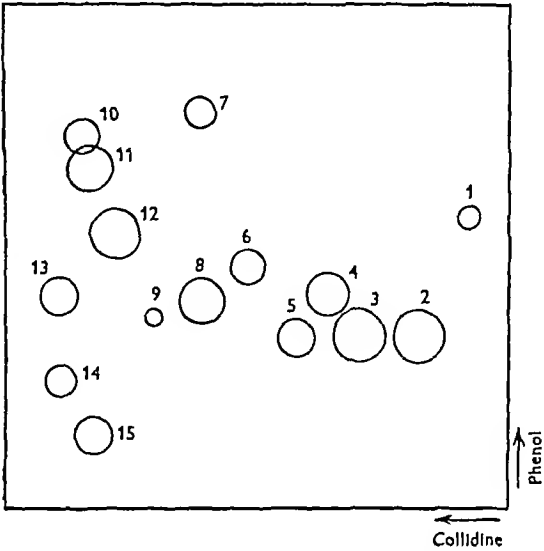


Fig 6 Diagram of chromatogram obtained from acid hydrolysate of Bence Jones protein An amount containing 50 μ g of N was taken and placed at the bottom right-hand corner of the filter paper Phenol was run from right to left followed by 'collidine' in an upward direction The latter was run for half as far again as the phenol The sizes of the spots have been drawn to represent the relative strengths of ninhydrin colour reaction according to an arbitrary scale The identifications are 1, cysteic acid (from cystine), 2, aspartic acid, 3, glutamic acid, 4, serine, 5, glycine, 6, threonine, 7, tyrosine, 8, alanine, 9, histidine, 10, phenylalanine, 11, leucine and/or isoleucine (an independent method showed that both were present), 12, valine, 13, proline, 14, arginine, 15, lysine

common amino acids except tryptophan (destroyed in acid hydrolysis), methionine, histidine and

Table 1 Analysis of Bence Jones protein

(All values are expressed as % (w/w) of dry ashless protein except in the case of values for ash itself which are corrected for dry material only)

	This paper	Calvery & Freyberg (1935)		Devine (1941)	Harvier & Rangier (1943)
		Sample 1	Sample 2		
Ash	0.16	5.1	1.2	0.4	—
Total nitrogen	16.2	18.0	18.1	14.7	—
Total sulphur	0.76	1.0	1.0	1.34	1.15
Cystine	2.47	3.0	3.0	2.9	—
Inorganic sulphur	0.10	—	—	—	—
Carbohydrate	0.17	—	—	—	—
Methionine (chemical methods)	0.00*	0.81†		0.58	1.80‡
Methionine (microbiological method)	<0.02	—	—	—	—
Glucose/nitrogen ratio	0.0108	—	—	—	0.07

* The gravimetric method for methionine gave a small negative value, cystine + inorganic sulphur averaging 0.768 (0.753, 0.771, 0.778, 0.776) and total sulphur 0.761 (0.760, 0.758, 0.765) The reason for this is not known

† This value does not appear in the paper by Calvery & Freyberg (1935), but is attributed to them by Block & Bolling (1947, p. 178)

‡ The authors do not indicate which chemical method was used The protein was obtained not from a case of myelomatosis, but from the urine of a patient with multiple secondary carcinoma of bone

hydroxyproline There is also a spot in the position occupied by leucine and/or isoleucine After oxidation with hydrogen peroxide the cysteic acid spot showed strongly, indicating the presence of a fair quantity of cystine, but no methionine sulphone was seen as would have been found had methionine been present in the original protein The same result was also obtained from a sample of the protein passed in the urine during the period of methionine administration The chromatograms run with 10 times the usual quantity showed histidine, but still no methionine as its sulphone, nor any hydroxyproline As the technique readily detects $5 \mu\text{g}$ of methionine there must have been less than $5 \mu\text{g}$ in the 3 mg of protein taken, i.e. less than $0.16 \text{ g}/100 \text{ g}$ of protein The presence of histidine in small amounts was confirmed by the one-dimensional collidine chromatogram, tryptophan by the Hopkins-Cole reaction, leucine and isoleucine (much more of the former) in the *tert* amyl alcohol chromatogram

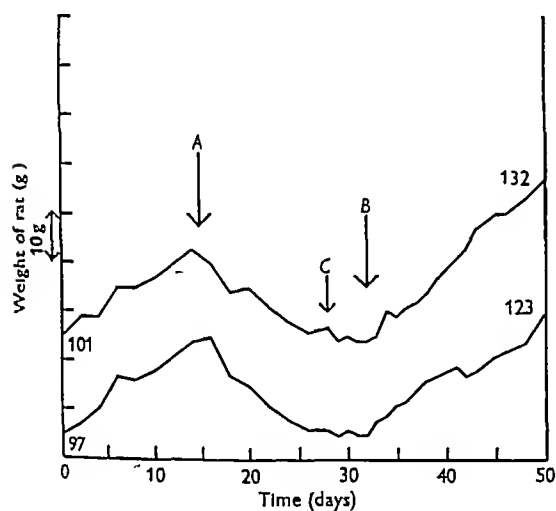


Fig 7 Growth curves in first rat experiment Before arrow A both rats received casein as sole source of protein in the diet From A till the end of the experiment Bence Jones protein containing the same amount of nitrogen was substituted for casein After arrow B methionine was added to the diet, between C and B α aminobutyric acid was added The weights of the two rats at the beginning and end of the experiment are marked on the curves

Chemical analyses (including microbiological) These are shown in Table 1

Biological results The growth curves from the two rat experiments are shown in Figs 7 and 8 In all cases rats given Bence-Jones protein diet without added methionine developed marked loss of appetite so that instead of eating quickly the maximum daily allowance of 11 g they ate only 7 or 8 g On the addition of methionine, appetite rapidly returned

DISCUSSION

The recognition of the protein in our case was based on the clinical diagnosis and on its giving the classical reactions on heating The salting-out and electrophoretic properties indicated that about 90 % of the material consisted of a single component Nearly all the protein was precipitated within the concentration range of 38–46 % saturation with regard to sodium sulphate (Figs 2 and 3) The salting-out curve with zinc sulphate of the less soluble fraction (Fig 4) fully confirmed that the upper point of inflexion was as sharp as has been drawn and that the upper part of the curve was a straight line

With regard to the chemical composition of the protein, the paper chromatograms (Fig 6) clearly displayed the many amino-acids present in the acid hydrolysate All the 'essential' or 'indispensable' amino-acids (Rose, 1938) were found with the

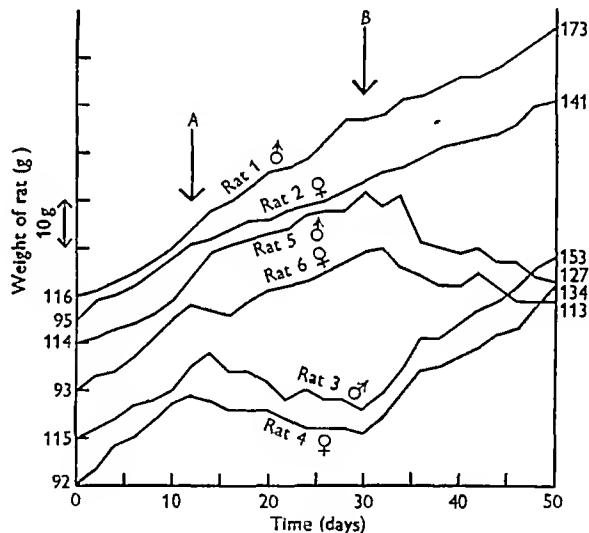


Fig 8 Growth curves in second rat experiment Before arrow A all rats received casein as sole source of protein Rats 1 and 2 continued to receive casein throughout the experiment After A and until the end of the experiment rats 3–6 received an equivalent amount of Bence-Jones protein instead of the casein Between arrows A and B rats 5 and 6 received the methionine supplement, at B the supplement was transferred to rats 3 and 4 The weights of the rats at the beginning and end of the experiments are shown on the curves The six rats were litter mates

notable exception of methionine The latter could not have been present to the extent of more than 0.2 % or it would have been detected by this highly specific technique In addition, the common 'non-essential' (or 'dispensable') amino acids were all found except hydroxyproline Compared with those obtained from a casein hydrolysate these chromatograms showed the presence of much higher relative amounts of serine and threonine In this feature the

protein shows some resemblance to the γ -globulin of human serum (Brand, Kassell & Saidel, 1944)

The feeding experiments with rats confirmed closely the chromatographic results. As would be expected from the complete absence of an essential amino acid the Bence-Jones protein failed to support growth or even to maintain the body weight of the animals. Supplementation, however, with the amount of methionine contained in the same weight of casein enabled growth to occur almost as well as in the control rats fed with casein as sole source of protein (Figs 7 and 8). The other essential amino-acids must therefore have been present in adequate amounts. Glynn, Himsworth & Neuberger (1945) describe experiments in which rats were fed pure amino acids in place of protein. Some of their diets were otherwise very similar to ours. They showed that a mixture of amino acids of the composition of a casein hydrolysate which provided daily only 8 mg of methionine, instead of the required 23 mg, allowed appreciable growth in the rats. The loss of weight on the unsupplemented Bence-Jones protein diet is therefore further evidence that there was much less than 8 mg of methionine in the daily allowance of about 0.8 g of protein, i.e. much less than 1% of methionine in the protein.

The two independent quantitative chemical methods also failed to detect any methionine in the protein. The microbiological assay led to the same result. More exactly, the latter showed that there could not have been more than 0.02% of methionine in the protein. If this maximal amount were actually present in the protein it would demand a minimum molecular weight of 650,000 for Bence-Jones protein which is far higher than the figure of about 35,000 generally accepted by most workers (Svedberg & Sjogren, 1929).

The material thus belongs to that small group of proteins devoid of methionine (e.g. insulin, tobacco mosaic virus) and differs conspicuously in this respect from the proteins present in normal plasma (Brand *et al.* 1944). Only three other analyses for methionine in Bence-Jones protein have been found in the literature (Table 1). The results of these do not agree with ours or with each other. The methods used are, however, either not mentioned or else are now not accepted as satisfactory, nor were any of the results checked by independent methods as in the present work. In view of these facts and of the possible differences between different samples of the protein, we think, therefore, that these results in no way detract from our findings that the Bence Jones protein, at least in our particular case, contains no methionine. All the other amino acids, except for the biologically unessential hydroxyproline, were present. Our findings support strongly the work of Abderhalden (1940) who claims, as have others before him, that Bence-Jones protein is quite distinct from

proteins of normal human plasma. The physico-chemical and immunological differences between different samples of the protein such as have been shown to occur by other workers (Hewitt, 1929, Abderhalden, 1940, Hektoen & Welker, 1940) still remain a puzzling feature. However, if the absence of methionine is confirmed in pure samples excreted by other patients a most definite point of similarity will be available, which may become useful for purposes of identification.

The above facts may be of significance in regard to the aetiology of the disease. The urinary protein has been shown to be present in the plasma of myeloma patients (Kydd, 1934, Moore, Kabat & Gutman, 1943, Blackman, Barker, Buell & Davis, 1944) and there is some evidence that the plasma cells may be the seat of its formation (Martin, 1947). In our case (and in one reported by Hewitt, 1929) there was a disease process at work resulting in the formation, by a very ill man, of at least 30–40 g/day of a fairly pure protein. The output of urinary protein was independent of changes in the diet and clinical condition of the patient, even being unaffected by adding to his diet 50 g of his own Bence-Jones protein, and it was maintained at this level over a period of months. For the reasons stated above, this protein was either foreign to the body or else present normally in only undetectably small quantities. Such a situation cannot be explained as an overaction of a normal process such as could occur as the result of excessive stimulation or of malignant change. For purposes of comparison, the rate of formation of other body proteins can be discussed. A healthy dog under the best conditions of diet and under maximal stimulus of hypoproteinaemia can produce some 10 g/day of mixed plasma proteins (Whipple, 1948). A human being with a liver weight 4 times greater could be expected to produce under similar optimal conditions 40 g/day. In severe nephrosis, the disease in which the largest proteinurias occur, it is rare for more than 30 g/day to be excreted and this may be largely serum albumin. The total protein formation in the regenerating rat liver, which grows much faster than most malignant tumours (Brues & Drury, 1936) is only about 60 mg/100 g body weight/day under the best conditions and during the peak growth period. At this rate the healthy human subject of 70 kg could produce about 40 g/day of liver proteins, but there is still no analogy here for the production of similar amounts of a single protein constituent. The other theories also put forward (summarized by Calvery & Freyberg, 1935) likewise cannot explain the facts.

The tentative suggestion is, therefore, put forward that multiple myelomatosis is due to invasion of the body by a virus which lives and multiplies in the plasma cells of the bone marrow. It is assumed to stimulate their growth as is the case with the white

cells in fowl leukaemia. It is further suggested that the Bence-Jones protein, when combined with nucleic acid in the plasma cells, is the virus itself.

This suggestion would be quite compatible with our knowledge of viruses. Tobacco mosaic virus, for instance, can multiply very rapidly almost up to the moment when the leaf dies, by which time the bulk of the total protein is in the form of virus protein (Stanley, 1937). Markham, Mathews & Smith (1948) have shown that the yellow mosaic virus growing in the turnip can produce protein free from nucleic acid as well as its own nucleoprotein. Such work as has been done on animal viruses shows a more complex mechanism as several proteins, physicochemically distinguishable from each other, may be produced by the one virus.

It may be relevant to allude here to the work of Snapper & Schneid (1946, 1947) and of Snapper, Mirsky, Ris, Schneid & Rosenthal (1947). They have shown that administration of stilbamidine to patients with myelomatosis may produce a temporary clinical improvement. During the treatment the plasma cells may show the presence of basophil inclusion bodies which they interpret as resulting from the reaction between the stilbamidine and a 'foreign nucleoprotein' present in the cells. It appears to the present writers that this could be the virus in question. Inclusion bodies of this type may also be found in proved virus diseases (Findlay & Ludford, 1926).

Further support for this theory comes from the fact that virus proteins, so far as they have been analyzed to date, are usually methionine free (Block & Bolling, 1947, p. 304; Chandler, Gerrard & du Vigneaud, 1947; Knight, 1947).

SUMMARY

1 A case of multiple myelomatosis is described in which Bence-Jones proteinuria was found.

2 The average daily output of protein was about 36 g. The output was not increased on adding 50 g of the protein to the usual diet.

3 A salting-out curve suggests that the protein consisted of two constituents in the proportion of about 90 and 10%. On electrophoretic analysis it gave one slightly asymmetrical peak which migrated with fibrinogen.

4 The protein was shown to be free of methionine by paper chromatographic, microbiological, biological and two different chemical methods. The hydrolysate of protein, on chromatographic analysis, showed the presence of all the other common amino-acids, except hydroxyproline. When supplemented with methionine, the protein was similar to casein in its ability to support the growth of rats.

5 A new spot test for cystine is described.

6 The qualitative and quantitative data presented are considered incompatible with the view that this Bence Jones protein is synthesized by the body tissues. Their similarity to those in certain virus diseases is indicated, and it is suggested that multiple myelomatosis could be explained as a virus infection and the abnormal urinary protein as a constituent protein of that virus.

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The Fate of Certain Organic Acids and Amides in the Rabbit

7 AN AMIDASE OF RABBIT LIVER

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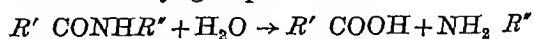
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In an earlier investigation in this laboratory (Bray, James, Ryman & Thorpe, 1948) it was shown that extracts of rabbit liver are capable of hydrolyzing the carbamyl group of several carboxylic acid amides, notably *p*-nitrobenzamide. The results of this study are summarized in Table 1. Subsequent

'amidases'. Since the most convenient substrates so far examined are *p*-nitrobenzamide and benzamide, it is convenient to refer to it provisionally as a benzamidase. It should be emphasized that the use of this term does not necessarily imply identity with the benzamide-synthesizing enzyme called benzamidase by Waelsch & Busztin (1937). We have found that rabbit liver extracts also hydrolyze certain aliphatic amides (e.g. propionamide (Table 1) and, more readily, butyramide and valeramide), but we have not yet sufficient evidence to say whether or not these compounds are hydrolyzed by the same enzyme as the aromatic amides.

It was, therefore, of interest to investigate the relationship of the benzamidase to the known amidases. Enzymes have been described which hydrolyze compounds containing a simple or substituted carbamyl group, thus



Thus glutaminase and asparaginase, which hydrolyze respectively the monoamides of glutamic and aspartic acids (glutamine and asparagine) are amidases acting upon substrates in which R' is H. Hippuricase (histozone) hydrolyzes hippuric acid ($R' = \text{C}_6\text{H}_5$, $R'' = \text{CH}_2 \text{ COOH}$) to benzoic acid and glycine. Deacetylases which hydrolyze acetamido compounds ($R' = \text{CH}_3$, $R'' = \text{C}_6\text{H}_5$, $\text{CH}_2 \text{ COOH}$, etc.) may also be regarded as amidases. There is the further possibility that some peptidases or proteases which hydrolyze the 'peptide' link might be capable of hydrolyzing amides. In the above mentioned study (Bray *et al.* 1948) it was observed that, although pepsin and trypsin were without action upon the amides studied, papain hydrolyzed hippuramide readily and benzamide to a slight extent.

Table 1 *Hydrolysis of amides by rabbit-liver extract at pH 7.4*

Amide	Percentage hydrolysis	
	After 10 hr	At equilibrium
Benzamide	24	45
<i>o</i> Hydroxybenzamide	0	0
<i>m</i> Hydroxybenzamide	0	1
<i>p</i> Hydroxybenzamide	3	6
<i>o</i> Aminobenzamide	5	9
<i>m</i> Aminobenzamide	2	7
<i>p</i> Aminobenzamide	3	13
<i>o</i> Nitrobenzamide	13	23
<i>m</i> Nitrobenzamide	15	31
<i>p</i> Nitrobenzamide	73	82
<i>o</i> Bromobenzamide	4	21
Formamide	0	0
Acetamide	1	2
Propionamide	14	25
Phenylacetamide	5	10
Nicotinamide	8	13
Asparagine	30*	—
Glutamine	22*	—

* Equilibrium reached

investigations have shown that, in most cases, and especially with aromatic amides, these results are in good agreement with those obtained with the intact animal (see Bray, Thorpe & Wood, 1949).

The enzyme responsible for this reaction may be included in the group of enzymes often termed

The literature dealing with these amidases is in some cases very extensive some of the findings of earlier workers are summarized in the following section

Asparaginase This enzyme has been shown to be present in tissues (e.g. calf liver, Suzuki, 1936), bacteria (e.g. Shibata, 1904, Dox, 1909, Utzino & Imaizumi, 1938) and yeast (Geddes & Hunter, 1928, Grassman & Mayr, 1933). The optimum pH as determined by some of these workers lies between 7 and 8.1. Geddes & Hunter stated that yeast asparaginase hydrolyzed glutamine, but not salicylamide, propionamide, acetamide or formamide. Grassman & Mayr showed that a partially purified preparation from the same source did not attack glutamine, acetamide or propionamide. Utzino & Imaizumi found that bacterial asparaginase deacylated some acyl derivatives of asparagine.

Glutaminase Krebs (1935) states that there is more than one glutaminase, differing principally in optimum pH's. One is found in brain and kidney and another in liver. Extracts of these tissues hydrolyze, but synthesis may occur in slices. The enzyme was found to be highly specific and would not hydrolyze asparagine. Further evidence to the effect that glutaminase and asparaginase are distinct enzymes was provided by the fact that the ratio of glutaminase activity to asparaginase activity was not constant for different tissues. Archibald (1944) also found that dog kidney had no action on asparagine.

Hippuricase (histozyme) Active preparations of this enzyme have been obtained from various tissues by Mutch (1912), Smorodnizew (1922), Tamura (1923-4), Takahashi (1929), Kimura (1928-9) and So (1930). It has also been obtained from takadiastase (e.g. by Blagowestschenski & Nikolaeff, 1935, Ellis & Walker, 1942). Smorodnizew's preparations hydrolyzed also homologues of hippuric acid and some other compounds including acetyl glycine while those of Tamura attacked dibenzoyl L-tyrosine and -L cystine. Kimura's preparations deacetylated acetyl glycine. So (1930) reported that his preparations did not hydrolyze benzamide, while those of Ellis & Walker were without action towards aromatic acetamido compounds and benzamide and acetamide.

Deacetylase Deacetylation by tissues *in vitro* has been demonstrated by several workers. Kohl & Flynn (1940) showed that rat liver will deacetylate N-acetylsulphanilamide. Michel, Bernheim & Bernheim (1937) showed that dog, cat, rabbit and ox liver and rat kidney readily deacetylated acetanilide. They concluded that the enzyme was not identical with hippuricase. Krebs, Sykes & Bartley (1947) found that pigeon liver and sheep kidney and liver deacetylated N⁴-acetylsulphamezathine. The experiments of Smorodnizew (1922) and Kimura (1928-9), on the deacetylation of aliphatic acetamido compounds *in vitro*, have already been mentioned.

Tissue peptidases and proteases Utzino (1928) found that pig and rabbit liver and pig kidney extracts hydrolyzed various dipeptides, including glycyl glycine. The chief tissue protease appears to be cathepsin (Willstätter & Bamann, 1929), which is a mixture of several enzymes, including peptidases (Fruton, Irving & Bergmann, 1941). Its optimum pH is 4-5 and it is believed to be inactive at pH 7.

Benzamidase Waelsch & Busztin (1937) reported the presence in horse kidney of an enzyme which synthesized benzamide from benzoic acid and ammonia at pH 7.3.

Borsook & Dubnoff (1940), however, were unable to demonstrate conjugation of benzoic acid under their conditions. Waelsch & Busztin also stated that benzamide was hydrolyzed by horse kidney.

Nicotinic acid amidase The hydrolysis of nicotinamide has been demonstrated in man by Johnson, Hamilton & Mitchell (1945) and in the guinea pig and rabbit by Ellinger & Abdel Kader (1948). The liver extracts which we used in our earlier study (Bray *et al.* 1948), hydrolyzed nicotinamide to the extent of 13% at equilibrium (see Table 1).

It is clear from the foregoing that the benzamidase with which we are concerned bears several points of resemblance to previously described enzymes. In this paper we report upon the distribution of some of these enzymes in the tissues of different species, together with their stability towards various treatments. From the results obtained it seems probable that the benzamidase is not identical with any of these enzymes.

METHODS

Materials The substrates used were benzamide (British Drug Houses Ltd.), p-nitrobenzamide (prepared from p-nitrobenzoyl chloride (British Drug Houses Ltd.) and ammonia), glutamine (isolated from mangolds by the method of Vickery, Pucher & Clark, 1935), asparagine (Ashe Laboratories, Ltd.), acetyl glycine (prepared by the method of Radenhausen, 1895), nicotinamide (British Drug Houses Ltd.) and glycyl glycine hydrochloride (prepared by the method of Fischer & Fournau, 1901). Phosphate buffer (0.2 M, pH 7.4) was used unless otherwise stated. The veronal buffer used in certain experiments contained sodium diethyl barbiturate (0.1 M, 5.81 vol.) and HCl (0.1 M, 4.19 vol.). Acid-washed sand (British Drug Houses Ltd.) was used in the preparation of tissue extracts.

Extracts The rat, rabbit or guinea pig was killed by a blow on the back of the neck and immediately bled. The organs required were removed, thoroughly ground with sand and then mixed with water, 1.5 times the original weight in the case of liver, 3 times with kidney and 5 times with brain. The resultant breis were centrifuged and the supernatant liquid decanted and used immediately. The cat and dog tissues were obtained from nembutal anaesthetized animals. The horse kidney arrived at the laboratory about 1 hr. after the death of the animal.

Digests Tissue extract (10 ml.) was added to the solution of the substrate (0.00687 M) in buffer (50 ml.). Chloroform was added as preservative and incubation carried out at 37.4°. In each experiment a control consisting of buffer and tissue extract without substrate was included.

Methods of estimation The hydrolysis of the substrates was determined by measurement of the increase in acidity using the formol titration technique previously described (Bray *et al.* 1948). In addition, wherever practicable, the progress of the reaction was also followed by estimation of the NH₃ liberated. This was done by transferring samples of digest (4 ml.) to a Folin type aeration apparatus, adding K₂CO₃ (5 g.) and blowing the NH₃ present into a known volume of standard acid (0.01 N). Back titration of the acid remaining, using de Wesselow's indicator (Cole, 1926), gave an estimate of the NH₃ liberated. Blank estimations were similarly made. Good agreement was found between the results

obtained by the two methods in the case of benzamide, *p* nitrobenzamide and nicotinamide, but under the conditions employed for the formol titration, glutamic and aspartic acids gave only 54 and 38% respectively of the theoretical increase in titration. Even when correction for this was applied, the formol titration for glutamine digests tended to give lower results for the degree of hydrolysis than the NH_3 estimation (see the following paper, Bray, James, Raffan & Thorpe, 1949). We have used the value obtained by NH_3 estimation as a measure of the degree of hydrolysis of glutamine and asparagine throughout this investigation.

Whilst earlier workers (Kimura, 1928-9, Michel *et al* 1937) used either acetyl glycine or acetanilide as substrate in determining deacetylase activity, we used both compounds in view of the probability of the existence of different mechanisms for aliphatic and aromatic compounds as already mentioned. The liberation of aniline from acetanilide was determined by a procedure similar to that of Bratton & Marshall (1939) for sulphanilamide in blood. Aniline sulphate was used as standard.

Control experiments In addition to those referred to above, control experiments were carried out in order to determine the stability of the substrates used to incubation at 37° and pH 7.4 for periods corresponding to those used in actual experiments. Only glutamine was appreciably unstable, undergoing slow spontaneous decomposition to an extent of about 28% in 20 hr (see Bray, James, Raffan & Thorpe 1949). This is in general agreement with the results of Hamilton (1945). The effect of this decomposition is negligible when dealing with tissue extracts having high glutaminase

activity, since the enzymic reaction is a rapid one, but in preparations with lower activity it may yield results which are significantly too high.

RESULTS

Enzymic activity of fresh tissues Table 2 shows the percentage hydrolysis of the various substrates by the tissues used in 3 and 20 hr. The values are the average results of the number of experiments indicated. As might be expected, there was considerable variation in the activities of the extracts of the organs of different individual animals. The averages give a reasonable indication of the consistent activity of the given tissue.

The effect of acetone treatment and precipitating agents on the enzymic activity of rabbit and dog liver

(1) **Acetone treatment of rabbit liver** (cf. Michel *et al* 1937). Rabbit liver (20 g) was ground with sand and water (30 ml). Acetone (150 ml) was then added, and, after standing for 1 hr, the suspension was filtered and the residue washed with acetone and dried in air. The product was then extracted with water (60 ml) for 1 hr at 0° in the presence of chloroform and the mixture centrifuged. Acetone was added to the supernatant liquid until precipitation was complete. The precipitate was separated at the centrifuge, washed with acetone and finally freed

Table 2 *Enzymic activity of various tissues at pH 7.4*

Average percentage hydrolysis of amides, with number of experiments in parentheses

Tissue	Time (hr)	<i>p</i> Nitro benzamide	Benzamide	Glutamine*	Asparagine	Acetyl glycine	Acetanilide	Nicotinamide	Glycyl glycine
Rabbit liver	3	(21) 42	(5) 15	(8) 46	(2) 32	(4) 44	(2) 23	(1) 0	(2) 56
	20	(24) 74	(5) 44	(10) 62	(5) 33	(4) 85	(4) 26	(2) 5	(2) 76
Guinea pig liver	3	(3) 5	(3) 5	(5) 20	(5) 61	(1) 100	(2) 28	(1) 2	— —
	20	(3) 11	(3) 14	(4) 38	(4) 79	(2) 100	(2) 79	(1) 2	— —
Rat liver	3	(6) 4	(6) 2	(5) 31	(3) 43	(4) 53	(2) 32	— —	(2) 18
	20	(6) 9	(6) 4	(5) 56	(3) 49	(4) 82	(3) 64	— —	(2) 25
Cat liver	3	(1) 4	(1) 2	(1) 3	(1) 7	(1) 24	(1) 3	— —	— —
	20	(1) 12	(1) 2	(1) 37	(1) 16	(1) 75	(1) 23	— —	— —
Dog liver	3	— —	— —	— —	— —	(2) 4	(3) 21	— —	— —
	20	(2) 35	— —	— —	— —	(3) 6	(3) 63	— —	— —
Rabbit kidney	3	(4) 13	(4) 0	(4) 55	(4) 0	(3) 65	— —	— —	(2) 81
	20	(4) 16	(4) 2	(2) 87	(4) 4	(3) 83	(3) 14	(2) 5	(2) 87
Guinea pig kidney	3	(3) 0	(3) 0	(4) 39	(4) 30	(1) 76	(2) 5	(1) 0	— —
	20	(3) 0	(3) 0	(5) 63	(4) 44	(2) 95	(2) 9	(1) 0	— —
Rat kidney	3	(3) 2	(2) 2	(2) 97	(2) 23	(2) 58	— —	(3) 1	— —
	20	(3) 1	(2) 3	(2) 97	(2) 24	(2) 97	(2) 13	(3) 9	— —
Cat kidney	3	(1) 0	(1) 0	(3) 70	(1) 2	(1) 31	(1) 23	— —	— —
	20	(1) 10	(1) 0	(3) 85	(1) 2	(1) 77	(2) 47	— —	— —
Dog kidney	3	— —	— —	— —	— —	(1) 35	(1) 9	— —	— —
	20	— —	— —	— —	— —	(2) 96	(1) 12	— —	— —
Horse kidney	3	— —	(1) 0	(1) 71	(1) 4	(1) 53	— —	— —	— —
	20	(1) 5	(1) 0	(1) 88	(2) 10	(1) 79	— —	— —	— —
Rabbit brain	3	(2) 0	— —	(5) 81	— —	— —	— —	— —	— —
	20	(2) 0	— —	— —	— —	— —	— —	— —	— —

* Values calculated from actual titrations, not corrected for spontaneous decomposition (see Bray, James, Raffan & Thorpe, 1949).

from solvent by leaving overnight *in vacuo* at ordinary temperature. In this way a fine powder (1.4 g) was obtained. Before use it was suspended in water (25 ml) and incubated at 37.4° for 1 hr. The activity of the preparation towards the various substrates was then determined in the usual way. The results are summarized in Table 3 which also shows the activity of the corresponding fresh tissue. It is evident that only the aliphatic deacetylase activity is retained to any appreciable extent.

(3) *Safranine precipitation* Fractionation of the liver extract by precipitation with safranine was carried out according to the method of Geddes & Hunter (1928). To the rabbit-liver extract (30 ml), prepared as previously described, an aqueous solution of safranine (30 ml, 0.5%) was added. The precipitate formed was separated by centrifuging and washed twice with water. It was then re-suspended in water (30 ml) and the activity of the suspension towards *p*-nitrobenzamide, benzamide,

Table 3 *Effect of acetone treatment and of precipitation on the enzymic activity of liver*

		Percentage hydrolysis of											
		<i>p</i> Nitrobenzamide		Benzamide		Glutamine		Asparagine		Acetylglycine		Acetanilide	
		3 hr	20hr	3 hr	20 hr	3 hr	20 hr	3 hr	20 hr	3 hr	20 hr	3 hr	20 hr
(1) Rabbit liver, acetone treatment													
Activity of fresh extract	(a)	62	72	—	—	17	47	—	25	62	69	35	42
	(b)	39	95	—	—	38	88	—	—	—	—	—	—
	(c)	—	—	—	—	—	—	37	37	50	98	8	18
Activity of dried liver	(a)	10	20	—	—	0	33	0	0	84	84	0	0
	(b)	10	10	—	—	0	28	—	—	—	—	—	—
	(c)	—	—	—	—	—	—	4	0	62	82	0	0
(2) Acetic acid precipitation													
Activity of fresh extract	(a)	44	86	—	—	—	—	—	—	—	—	—	—
	(b)	55	100	—	—	—	—	—	—	—	—	—	—
	(c)	31	100	12	24	29	58	—	51	—	—	—	—
	(d)	—	80	—	40	—	78	—	30	62	91	—	—
	(e)	—	—	—	—	38	88	—	—	—	—	—	—
	(f)	—	—	—	—	76	100	43	35	—	—	—	—
Activity of precipitate	(a)	43	79	—	—	—	—	—	—	—	—	—	—
	(b)	57	100	—	—	—	—	—	—	—	—	—	—
	(c)	42	79	10	19	12	40	—	37	—	—	—	—
	(d)	—	88	—	44	—	49	—	10	0	67	—	—
	(e)	—	—	—	—	7	36	—	—	—	—	—	—
	(f)	—	—	—	—	51	100	43	49	—	—	—	—
(3) Safranine precipitation													
Activity of fresh extract	(a)	40	47	14	17	24	100	24	33	—	—	—	—
	(b)	33	75	6	18	76	100	43	35	—	—	—	—
Activity of precipitate	(a)	33	59	2	30	21	73	23	36	—	—	—	—
	(b)	36	53	7	13	23	99	46	49	—	—	—	—
Dog liver													
Activity of fresh extract		—	—	—	—	—	—	—	—	2	15	22	57
Activity of acetone treated liver		—	—	—	—	—	—	—	—	0	0	0	0

* Values calculated from actual titrations, not corrected for spontaneous decomposition (see Bray, James, Raffan & Thorpe, 1949)

(2) *Acetic acid precipitation* (cf. Geddes & Hunter, 1928) The aqueous rabbit-liver extract, prepared as previously described, was adjusted to pH 4.5 by the addition of acetic acid (using British Drug Houses Ltd '4.5' indicator). The precipitate formed was separated by centrifuging, washed with water and suspended in a volume of water equal to that of the original extract. The activity of this suspension towards the substrates was then determined. The results are given in Table 3. It can be seen that the precipitate retained the benzamidase activity together with the ability to hydrolyze glutamine and asparagine.

glutamine and asparagine was compared with that of the fresh extract. Ammonia estimations were used since the colour of the digests interfered with the end point of the formol titrations. The results are recorded in Table 3. It is clear that the safranine precipitate retained almost completely all the enzymic properties investigated.

(4) *Acetone-treated dog liver* This was prepared in the same way as the rabbit-liver preparation. It is seen that the aromatic deacetylase activity is destroyed by acetone treatment as in rabbit liver. Unlike rabbit liver, dog liver has feeble aliphatic deacetylase activity even when fresh.

Hippuricase activity The hippuricase activity of rabbit liver was examined by incubating the extract (10 ml) with 0.00687M-hippuric acid in phosphate buffer pH 7.4 (50 ml). Samples were withdrawn at intervals and the acidity measured by means of a formol titration. In each experiment the benz-

0.00687M solutions (50 ml) of *p*-nitrobenzamide, glutamine, asparagine and acetylglycine in phosphate buffer pH 7.4. A second similar set of digests was set up with rabbit liver extract which had been heated at 50° for 30 min. The results are given in Table 5.

Table 4 *Enzymic activity of dried hog kidney*

Time (hr)	Percentage hydrolysis of						
	<i>p</i> Nitrobenzamide	Benzamide	Glutamine	Asparagine	Hippuric acid	Acetylglycine	Acetanilide
19	—	0	—	0	60	75	15
23	0	—	28*	0	—	—	—
44	16	0	—	—	71	79	—
68	—	—	—	—	92	—	—

* This value would be accounted for by the spontaneous decomposition of glutamine (see Bray, James, Raffan & Thorpe, 1949).

amidase activity was determined by incubating a digest containing *p*-nitrobenzamide. No hydrolysis of hippuric acid occurred in 48 hr using extracts which hydrolyzed *p*-nitrobenzamide to extents varying from 48 to 86% in 24 hr. Whilst the optimum pH of tissue hippuricase has been reported as 6.4–7.2 (Kimura, 1928–9) and 6.8–7.0 (So, 1930), that from takadiastase, which has frequently been used as a source of hippuricase, has been recorded as 5.4–5.8 (Ellis & Walker, 1942), and 8.0 (Blagowestschenski & Nikolaeff, 1935). Rabbit liver extract was, therefore, also incubated with hippuric acid in buffer solution pH 5.9, but no hippuricase activity could be detected. (Actually we could detect no hippuricase activity in the specimens of takadiastase (Parke, Davis and Co.) available to us.) It was found, however, that dried hog kidney constituted a convenient source of hippuricase: the material used was a desiccated and defatted commercial preparation (Viobin Corp., Monticello, Illinois, U.S.A.). The preparation (10 g) was incubated with water (100 ml) overnight at 37.4° in the presence of chloroform. The filtered extract (10 ml) was then added to 0.00687M-solutions of each of the substrates in phosphate buffer (50 ml) pH 7.4. A mixture of extract (10 ml) and buffer (50 ml) was used as control. Incubation of the digests was carried out in the usual way and the course of the reaction followed by formol titrations and ammonia estimations where appropriate. The results of a typical experiment are given in Table 4; other experiments gave similar results. It is evident that the extract of dried hog kidney possessed marked hippuricase and aliphatic deacetylase activity. It hydrolyzed acetanilide to a slight extent, but had negligible hydrolytic effect on *p*-nitrobenzamide, benzamide, glutamine and asparagine.

Effect of heat on enzymic activity of rabbit-liver extract Rabbit-liver extract (10 ml) was added to

The benzamidase and asparaginase activities were not greatly affected by the heat treatment: the aliphatic deacetylase activity appeared to be reduced to some extent, since hydrolysis of acetylglycine proceeded more slowly with the heated extract, but glutaminase activity was completely destroyed. The values of 25 and 27% hydrolysis in Table 5 are regarded as being due to spontaneous decomposition (see Bray, James, Raffan & Thorpe, 1949).

Table 5 *Effect of heating at 50° on the enzymic activity of rabbit liver extracts*

(F=fresh extract, H=heated extract)

Time (hr)	Percentage hydrolysis of							
	<i>p</i> Nitro benzamide		Glutamine*		Asparagine		Acetyl glycine	
	F	H	F	H	F	H	F	H
Exp 1								
1	20	9	2	2	28	17	28	0
20	37	49	41	25	32	24	90	100
Exp 2								
1	23	20	—	0	12	6	30	17
3.5	24	24	5	10	14	28	71	50
21	41	27	42	27	—	34	100	100

* These values have not been corrected for spontaneous decomposition (see Bray, James, Raffan & Thorpe, 1949).

Benzamidase and glutaminase activity of rabbit-liver extracts in phosphate and veronal buffers at pH 7.4 Greenstein and his collaborators (Carter & Greenstein, 1947; Greenstein & Leuthardt, 1948) have reported that the phosphate ion enhances the activity of liver glutaminase. We have found that benzamidase is also more active in phosphate buffer. The results of two experiments comparing the activity of benzamidase and of glutaminase in phosphate and veronal buffers at pH 7.4 are shown in Table 6.

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Table 6 *Benzamidase and glutaminase activity of rabbit liver in phosphate and veronal buffers at pH 7.4*

Exp no	Substrate	Buffer	Percentage hydrolysis* after			
			2 5 hr	5 hr	10 hr	20 hr
1	<i>p</i> Nitrobenzamide	Veronal	8	12	20	28
		Phosphate	19	30	42	43
	Glutamine	Veronal	0	4.5	11	17
		Phosphate	11	23	35	41
2	<i>p</i> Nitrobenzamide	Veronal	13	21	23	23
		Phosphate	28	48	68	82
	Glutamine	Veronal	8	16	20	20
		Phosphate	33	54	79	98

* These values have not been corrected for spontaneous decomposition (see Bray, James, Raffan & Thorpe, 1949)

DISCUSSION

The results of the foregoing experiments are summarized in Tables 7 and 8. The symbols used to indicate the degree of enzymic activity must perforce be somewhat arbitrary. They have been allotted taking into consideration all the results

available. The symbol 0 is used for results which are within the experimental error of the titrations (usually less than 5%), ? for results which are very low and of doubtful significance (usually under 15% hydrolysis in 20 hr), + for results above this and below 40% hydrolysis, and ++ for results over 40%. On this basis Table 7 shows the distribution of the various enzymes in the tissues examined. It is clear that rabbit liver, and, to a lesser extent, dog liver are the only ones of those studied which effect considerable hydrolysis of *p*-nitrobenzamide or benzamide. The benzamidase activity of guinea pig, rat and cat livers is very slight. Enzymes which hydrolyse asparagine, acetylglycine and acetanilide to a considerable extent are present in rabbit, guinea pig and rat livers. The glutaminase activity of rabbit liver is greater than that of the others and on the basis of distribution it might be considered identical with benzamidase. The distribution of activity in the kidney extracts is, however, different. Glutaminase is present in all the fresh extracts, whereas the benzamidase activity is feeble or absent. It seems reasonable to conclude that benzamidase is not identical with asparaginase and the deacetylases of

Table 7 *Enzymic activity of extracts of liver, kidney and brain*

(0 indicates negligible, ? low, + moderate and ++ high activity)

Substrate	<i>p</i> Nitrobenzamide	Benzamide	Glutamine*	Asparagine	Acetyl-glycine	Acet-anilide	Hippuric acid	Nicotin-amide	Glycyl-glycine	DL Alanine
Extract										
Rabbit liver	++	++	++	++	++	+	0	?	++	0
Guinea pig liver	?	?	+	++	++	++		0		
Rat liver	?	?	+	++	++	++			+	
Cat liver	?	0	?	+	++	+				
Dog liver	+				?	++				
Rabbit kidney	+	0	++	0	++	?		?	++	0
Guinea pig kidney	0	0	+	++	++	?		0		
Rat kidney	0	0	++	+	++	?		?		
Cat kidney	?	0	++	0	++	++				
Dog kidney					++	?				
Hog kidney (dried)	?	0	0	0	++	+	++			
Horse kidney	0	0	++	?	++					
Rabbit brain	0		++							

* Spontaneous decomposition has been taken into account (see Bray, James, Raffan & Thorpe, 1949)

Table 8 *Enzymic activity of liver after various treatments*

(Symbols as in Table 7)

Treatment of rabbit liver	Effect upon					
	<i>p</i> Nitrobenzamide	Benzamide	Glutamine*	Asparagine	Acetylglycine	Acetanilide
Fresh extract	++	++	++	++	++	+
Acetone treatment	?		0	0	++	0
Acetic acid precipitate	++	++	++	++	++	
Safranine precipitate	++	++	++	++	++	
Heated at 50° for ½ hr	++		0	++	++	
Treatment of dog liver						
Fresh extract					?	++
Acetone treatment					0	0

* Spontaneous decomposition has been taken into account (see Bray, James, Raffan & Thorpe, 1949)

liver and kidney or with the glutaminase of kidney. Evidence for the non-identity of benzamidase and brain glutaminase is provided by the fact that brain extracts of high glutaminase activity do not hydrolyze *p* nitrobenzamide.

Table 8 summarizes the effect of various treatments upon the enzymic activities of rabbit liver. The precipitation methods have clearly failed to distinguish between benzamidase, glutaminase and asparaginase, but acetone treatment and heating at 50° have a more marked effect in reducing glutaminase activity than that of benzamidase. This is in agreement with Archibald's (1944) observation that glutaminase was almost completely destroyed by heating at 50° for half an hour and differentiates benzamidase from liver glutaminase.

With regard to the possible identity of benzamidase with hippuricase, we were unable to detect any hippuricase activity at either pH 5.9 or 7.4 in rabbit liver extracts which showed strong benzamidase activity. Further, a commercial preparation of dried hog kidney which possessed considerable hippuricase activity failed to hydrolyze *p*-nitrobenzamide or benzamide to any significant extent. In one experiment with horse kidney we were unable to detect under our conditions any hydrolysis of benzamide. Waelsch & Busztin (1937) found hydrolysis of benzamide by glycerol extracts of horse kidney. We did not attempt to repeat these authors' experiments on the synthesis of benzamide.

The very feeble activity of rabbit liver towards nicotinamide suggests that nicotinic acid amidase is not benzamidase. The experiments with glycylglycine showed that, whereas rabbit liver was very active in hydrolyzing this dipeptide, rabbit kidney, which had only feeble benzamide activity, was as active towards glycylglycine as was rabbit liver. This suggests that benzamidase differs from dipeptidase. The failure of the rabbit-liver extracts to liberate ammonia from DL-alanine may be interpreted as evidence against a deaminase being concerned in the reaction.

In a previous paper (Bray *et al.* 1948) we showed that, while pepsin and trypsin had no action on benzamide, commercial papain (which had no dipeptidase activity towards acetylglutamine) hydrolyzed the amide to some extent. Cathepsin, which

is found especially in liver and kidneys, is stated to be inactive at pH 7.4, acting best between pH 4 and 5. This renders it improbable that benzamidase activity is due to this protease. The amount of acid or ammonia liberated from our control digests was very small, indicating that there was little protease activity at pH 7.4.

In view of the fact that exact quantitative comparison of activities is not possible in experiments of this type, it seems justifiable to conclude that the enzyme benzamidase is not identical with any of those considered.

Another point of interest arising from these experiments is the indication of the existence of two deacetylases, one acting upon acetylglutamine and another upon acetanilide. In liver the distribution of the two enzymes is similar, except in the dog, which has little aliphatic deacetylase. In kidney, however, there is clearly greater aliphatic deacetylase activity, except in the case of the cat, which has also a high aromatic deacetylase activity. The distribution of the two enzymes in dog kidney is the reverse of that in the liver. This is in accordance with the suggestion made by several workers that the biological acetylation processes for aromatic and aliphatic amino compounds are different since the dog is able to acetylate the latter but not the former (e.g. Stekol, 1938; Marshall, Cutting & Emerson, 1937).

SUMMARY

1 A study has been made of the enzymic activity of liver and kidney extracts from various animals towards benzamide, *p* nitrobenzamide, asparagine, glutamine, nicotinamide, glycylglycine, acetylglutamine and acetanilide.

2 From the results obtained it appears probable that the enzyme responsible for the hydrolysis of the aromatic amides is not identical with any of those which attack the other substrates.

3 There is evidence for the existence of distinct aliphatic and aromatic deacetylases.

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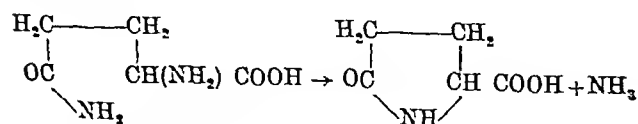
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The Enzymic Hydrolysis of Glutamine and its Spontaneous Decomposition in Buffer Solutions

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The investigation of glutaminase activity reported in the preceding paper (Bray, James, Raffan, Ryman & Thorpe, 1949) led us to consider the nature of the products of the action of glutaminase upon glutamine. The evidence hitherto presented for these being glutamic acid and ammonia is indirect and based upon ammonia and amino nitrogen estimations (e.g. Krebs, 1935, Archibald, 1944, Archibald & Hamilton, 1945). As far as we are aware, glutamic acid has not yet been isolated from the digests and characterized. Early in our present investigation it seemed possible that pyrrolidonecarboxylic acid might be a product of the reaction since there was a discrepancy between the percentage hydrolysis of glutamine brought about by tissue extracts as measured by formol titration (corrected) and by ammonia estimation. Such a result could be accounted for by the formation of pyrrolidonecarboxylic acid, which would give rise to the liberation of ammonia without an equivalent increase in formol acidity.



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This would have been in accord with the hypothesis of Leuthardt (1940), which was, however, withdrawn owing to failure to isolate pyrrolidonecarboxylic acid from digests.

The present paper shows that, under certain conditions, pyrrolidonecarboxylic acid may be formed in glutamine digests as a result of spontaneous decomposition.

EXPERIMENTAL

The methods and materials used were the same as those described in the preceding paper (Bray *et al.* 1949).

Estimation of amino N Samples from the digests were deproteinized with sodium tungstate and H_2SO_4 , and the amino N estimated in portions of the protein-free solution in the Van Slyke micro apparatus (reaction time 4 min). Under these conditions 90% of the N of glutamine was liberated and 40% of the N of ammonia (Hamilton (1945) found 38% under similar conditions). Ammonia N was estimated on another portion and the appropriate correction made to the amino N values.

RESULTS

Formol titration of glutamine, glutamic acid and pyrrolidonecarboxylic acid The compounds were dissolved in phosphate buffer pH 7.4. Glutamine and

pyrrolidonecarboxylic acid in concentrations up to 200 mg/100 ml gave the theoretical titrations corresponding to one carboxyl group/mol. Glutamic acid, however, in concentrations up to 300 mg/100 ml gave consistently low titrations corresponding to $77 \pm 3\%$ of the theoretical, calculated as equivalent to two carboxyl groups. In enzyme studies the conversion of glutamine to glutamic acid is measured, so that the increase in titration corresponds to the formation of a second carboxyl group. The first carboxyl group of glutamic acid can be taken as titrating normally, as in glutamine, so that the second carboxyl group only titrated to an extent of $54 \pm 6\%$. This value has been used to 'correct' the formol titrations. A higher percentage of the theoretical titration could be obtained by increasing the pH of the end point by using thymolphthalein instead of thymol blue as indicator, but the end point with the digests was so difficult that consistent duplicates could not be obtained. The ethanol titration method (Harris, 1923) was not practicable, owing to the large dilution with ethanol required.

Incubation of glutamine with tissue extracts Extracts of various tissues were incubated with glutamine in phosphate buffer at pH 7.4, and the extent of hydrolysis of the substrate estimated by both ammonia and formol methods. Table 1 shows some typical results. The formol values have been 'corrected' as described above. It can be seen that, in experiments in which glutamine was rapidly and almost completely hydrolyzed by the very active brain extracts (Exps 1 and 2), there was, considering the unprecise nature of the correction, reasonable agreement between the formol and ammonia values. In short term experiments with less active extracts (Exps 3 and 4), in which hydrolysis was far from complete, there was an indication that the ammonia values exceeded the formol values after 4–5 hr to a significantly greater extent than after 1 hr. This is shown more clearly in the long experiments (nos 5 and 6). In Exp 5 almost complete decomposition was attained after 23 hr as judged by the ammonia value, although the formol method indicated only 63% conversion. In Exp 6

with a less active extract the ammonia value after 22 hr was more than twice the formol value, although the extent of the discrepancy in the two experiments was the same (equivalent to 28% of the glutamine). This amount was the same as that obtained when glutamine was incubated with boiled tissue extract (Exp 7), and corresponded closely with the extent of the spontaneous decomposition of glutamine in phosphate buffer as described below.

Table 1 *Results of incubation of glutamine with extracts of various tissues in phosphate buffer pH 7.4*

Exp no	Tissue	Time (hr)	Percentage hydrolysis determined by	
			Formol ('corrected') method	Ammonia method
1	Rabbit brain	0.75	76	86
		4	76	88
2	Rabbit brain	0.5	61	60
		2.5	81	90
3	Rabbit kidney	0.5	11	23
		1.5	39	43
		4	46	66
4	Rabbit liver	0.67	9	14
		5	26	46
5	Rabbit kidney	3.5	54	61
		23	63	91
6	Rat liver	2	19	23
		4	19	29
		22	19	47
7	Rat liver (extract as in Exp 6, heat-inactivated)	2	0	5
		22	0	30

Spontaneous decomposition of glutamine in buffer solutions It is known that glutamine in neutral, weakly acid or alkaline solution is readily converted to pyrrolidonecarboxylic acid by heating at 100° (Chibnall & Westall, 1932, Vickery, Pucher, Clarke, Chibnall & Westall, 1935, Melville, 1935). This reaction at pH 6.5 and 100° is accelerated by the presence of phosphate (Hamilton, 1945). It seemed desirable, therefore, to examine the effect of keeping glutamine in a phosphate solution at 37° .

Table 2 *Spontaneous decomposition of glutamine in buffer solutions*

pH	Buffer	Glutamine in substrate (M)	Percentage decomposition after				
			2.5 hr	5 hr	10 hr	20 hr	40 hr
7.4	Phosphate	0.005706	7.0	11.0	17.0	26.5	40.5
7.4	Phosphate	0.01141	7.0	9.2	12.4	26.0	41.0
7.4	Phosphate	0.02282	4.5	9.2	16.0	28.0	—
7.4	Phosphate	0.01364	7.0	12.5	20.3	32.0	46.5
6.8	Phosphate	0.01141	2.5	5.0	10.0	19.6	39.5
8.0	Phosphate	0.01141	7.0	12.5	22.0	37.0	56.8
7.4	Veronal HCl	0.01370	0.0	0.1	1.2	3.8	10.4
7.4	Veronal HCl	0.02740	2.0	3.0	5.0	8.8	14.8
Average for phosphate buffer, pH 7.4			6.5	10.5	18.5	28.0	43.0
Average for veronal buffer, pH 7.4			1.0	1.5	3.1	6.4	12.6

Solutions of glutamine were incubated in buffer solutions at 37.4°. Samples were withdrawn at intervals, and the extent of decomposition measured by estimation of ammonia, there was no increase in formol acidity. The results are given in Table 2.

It is clear that on long incubation there is appreciable decomposition of glutamine. This is greater in phosphate buffer, in which approximately 28% of glutamine is decomposed in 20 hr, than in a phosphate-free buffer (veronal), in which only 6% is decomposed. The absence of increase in formol acidity suggests that pyrrolidonecarboxylic acid is the product. This we have confirmed by isolation of the compound from a solution of glutamine (400 mg) incubated in phosphate buffer (100 ml) for 42 hr. The acid was extracted with ethyl acetate (cf Woodward & Reinhart, 1942) and had m.p. 157–160° melting point mixed with authentic pyrrolidonecarboxylic acid, 157–160°.

Attempted isolation of end product of the action of liver extracts on glutamine. For these experiments short-term digests were used so as to avoid the complication of the formation of considerable amounts of pyrrolidonecarboxylic acid by spontaneous decomposition of glutamine.

We were unable to detect any pyrrolidonecarboxylic acid in our digests by the method used successfully

by Woodward & Reinhart (1942) for its isolation from digests of glutathione. Several estimations of the loss of amino nitrogen supported Krebs's (1935) findings that glutamic acid and not pyrrolidonecarboxylic acid is formed. Attempts to isolate glutamic acid from glutamine digests have so far been unsuccessful, but paper chromatography (cf Consden, Gordon & Martin, 1944) of the material extracted by *n*-butanol from such digests suggests that the product is indeed glutamic acid. We thus have no reason to dispute the conclusion of the earlier workers that glutamic acid is the principal product of the enzymic hydrolysis of glutamine.

SUMMARY

The nature of the products of the action of tissue extracts on glutamine has been examined. Whilst glutamic acid appears to be the main product, significant amounts of pyrrolidonecarboxylic acid may be present in long-term digests, owing to spontaneous decomposition of glutamine.

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The Effect of Various Diets on the Metabolism of Nicotinic Acid and Nicotinamide in the Rabbit

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It has recently been shown (Ellinger & Abdel Kader, 1948, 1949) that nicotinic acid and nicotinamide are not metabolized in an identical manner by various mammalian species. Man, dog, cat and rat aminate nicotinic acid to nicotinamide and methylate the latter to nicotinamide methochloride, whilst the rabbit and guinea pig deaminate nicotinamide to nicotinic acid and eliminate small amounts of an

unidentified compound which was provisionally called 'pseudo nicotinamide methochloride'. Whilst the guinea pig eliminates nicotinic acid unchanged, the rabbit methylates part of it to trigonelline. It appears that the species which aminate nicotinic acid are omnivorous or carnivorous, while those which deaminate nicotinamide are strictly herbivorous. It appeared possible that these differences

are related to the type of diet and not to the constitution of the species. In order to decide which was the real cause, the work described in this paper was carried out. Rabbits were chosen as test objects for the following reasons. It can be expected that a change in diet will affect the omnivorous less than the strictly herbivorous animals, and, among the latter, the rabbit has shown a more complex metabolism than the guinea pig. In addition, it is known that rabbits can be trained to eat meat (Aberhalden & Brahm, 1909).

regained 330 and 390 g, respectively, during the meat period and at the end their weights were 2730 and 2753 g respectively. At the end of the second cabbage period their weights were 2350 and 2420 g respectively. Without extradietary intake of either acid or amide the only metabolites detected were nicotinic acid and 'pseudo nicotinamide methochloride' (Table 2), as was found in the earlier experiments (Ellinger & Abdel Kader, 1949). The amounts of nicotinic acid eliminated were lowest during the cabbage, somewhat higher during the oat

Table 1 *Protein, fat, carbohydrate, nicotinic acid and nicotinamide contents and caloric values of the diets used*

Diet		Protein		Fat		Carbohydrate	
Type	(g/day)	(g/100 g)	(g/day)	(g/100 g)	(g/day)	(g/100 g)	(g/day)
Cabbage	200	1.6*	3.2	0.3*	0.6	5.6*	11.2
Rolled oats	100	16.7*	16.7	7.3*	7.3	66.2*	66.2
Meat meal +	50	64.2§	32.1	26.4§	13.2	—	—
national bread	50	8.5	4.25	1.2	0.6	57.4	28.7
		36.35		13.8		28.7	

Diet		Calories		Nicotinic acid	Nicotinamide
Type	(g/day)	(per 100 g)	(per day)	(mg/day)	(mg/day)
Cabbage	200	31.6†	63.2	0.204†	0.392†
Rolled oats	100	399.5†	399.5	0.530†	0.720†
Meat meal +	50	503.2†	251.6	1.250†	1.492†
national bread	50	250.0	125.0		
		376.6			

* From Tibbles (1912)

† Calculated (protein, 4.0 cal, fat, 9.3 cal, carbohydrate, 4.0 cal)

‡ Determined

§ Analysis supplied by Mr F. C. Hymas, Spratt Patent Ltd

|| From Medical Research Council (1945)

EXPERIMENTAL

Two male rabbits were kept in metabolism cages at 22–27°. The urine was collected every 24 hr and analyzed for nicotinic acid, nicotinamide, trigonelline and 'pseudo nicotinamide methochloride' as described (Ellinger & Abdel Kader, 1949). The rabbits were fed on cabbage alone for 3 weeks, on rolled oats for 4 weeks, on national bread and meat meal for 29 days and again on cabbage for another 22 days. Oats, bread and meat meal were moistened with water.

When the daily urinary output of the various metabolites was approximately constant for at least 5 days, nicotinic acid or nicotinamide (100 or 200 mg) was injected intraperitoneally at intervals of 3–5 days. The increase in metabolite elimination following the extradietary administration of the compounds was calculated by deducting the average daily predosing output from that found after administration of the compound. In all cases the predosing level was reached again after 24 hr. In two experiments choline chloride (0.15 g) was injected intraperitoneally together with 0.2 g of nicotinic acid.

RESULTS

Both rabbits reacted similarly to changes in the diet. On the cabbage diet the rabbits kept their weight of 2810 and 2850 g respectively. They lost 540 and 560 g respectively, during the oat period, they

and highest during the meat period, in accordance with the daily dietary intake of nicotinic acid and

Table 2 *Daily urinary elimination of nicotinamide metabolites by rabbits kept on different diets*

(Values for 'pseudo nicotinamide methochloride' are expressed in terms of nicotinamide methochloride.) (Neither nicotinamide, nicotinic acid nor trigonelline were eliminated.)

Diet	Rabbit no	Days	Metabolites eliminated (mg/day)	
			Nicotinic acid	'Pseudo nicotinamide methochloride'
Cabbage	1	5	0.793	0.501
	2	5	0.577	0.537
Oats	1	5	0.992	0.565
	2	5	0.983	0.500
Meat bread	1	8	1.755	0.619
	2	8	1.770	0.645

nicotinamide during each of these periods. The amount of 'pseudo nicotinamide methochloride' eliminated did not change markedly with the diet. Since the nature of this compound is unknown it could not be decided whether the output of meta-

bolites exceeded the intake of the compounds, indicating biosynthesis

As in the earlier experiments (Ellinger & Abdel Kader, 1949) on cabbage or oat diet extradietary nicotinic acid was nearly completely eliminated, but nicotinamide was eliminated to a much lesser extent, particularly after doses of 100 mg. The meat diet did not markedly affect the total metabolite output with the exception that after 100 mg amide, output was considerably higher than on either of the other diets (Table 3)

Both the ability to methylate and deaminate were, therefore, affected during the meat period. To obtain information as to whether this was due directly to the kind of food taken, or to changes caused by the diet on the tissues responsible for the metabolism, the metabolism of both nicotinic acid and nicotinamide was studied at different times after changing from the meat to the cabbage diet (Table 5). On the eighth day after the change, total output was still low, methylation was still absent and deamination as low as during the meat period. Methylation to

Table 3 *Effect of diet on increase in the total urinary elimination of nicotinamide metabolites (except 'pseudo nicotinamide methochloride') following the extradietary administration of nicotinic acid or nicotinamide*

(Figures are given as percentage of administered dose)

Compound administered		Total elimination							
		Rabbit 1				Rabbit 2			
		Nicotinic acid		Nicotinamide		Nicotinic acid		Nicotinamide	
		100	200	100	200	100	200	100	200
Dose (mg)									
Diet									
Cabbage		99.1	93.0	23.5	78.4	81.7	95.1	28.1	70.5
Oat		95.0	96.8	18.0	70.0	84.9	97.1	22.8	63.6
Meat-bread		92.1	96.3	36.5	55.3	76.0	99.3	43.2	61.9

Table 4 *Effect of diet on increase in the urinary elimination of individual nicotinamide metabolites (except 'pseudo nicotinamide methochloride') following the extradietary administration of nicotinic acid or nicotinamide*

(Figures are given as percentage of administered dose)

Compound administered		Metabolite eliminated (%)							
		Rabbit 1				Rabbit 2			
		Nicotinic acid		Nicotinamide		Nicotinic acid		Nicotinamide	
		100	200	100	200	100	200	100	200
Dose (mg)									
Metabolite eliminated	Diet								
Nicotinic acid	Cabbage	50.4	64.0	11.0	18.0	59.9	69.3	20.3	33.0
	Oats	45.3	62.4	9.5	17.2	63.7	74.3	17.9	30.3
	Meat-bread	92.1	96.3	14.1	7.8	76.0	99.3	16.0	12.7
Nicotinamide	Cabbage	0	0	2.6	17.7	0	0	0	0
	Oats	0	0	0.6	12.7	0	0	0	0
	Meat-bread	0	0	22.4	47.6	0	0	27.2	49.2
Trigonelline	Cabbage	48.5	29.0	9.9	42.7	25.8	25.7	7.8	36.6
	Oats	49.6	34.4	7.9	40.2	21.1	22.7	5.8	33.3
	Meat-bread	0	0	0	0	0	0	0	0

On a cabbage or oat diet, nicotinic acid injection was followed by the output of nicotinic acid and trigonelline only, this was also the case in one rabbit after nicotinamide injection, whilst the other eliminated in addition small amounts of unchanged nicotinamide (Table 4). During the meat period the results were very different, nicotinic acid was entirely eliminated as such and nicotinamide mostly unchanged, but to a small degree deaminated to the acid. Methylation was completely absent. There was no methylation either when choline was injected together with the acid

a reduced extent began again on the eleventh day, but methylation and deamination had not yet reached the pre-meat level on the sixteenth day of the cabbage diet. On the twenty-first day the pre-meat level was almost attained in both respects.

The output of 'pseudo nicotinamide methochloride' after extradietary nicotinic acid administration was hardly affected by the meat diet, that after nicotinamide application was, however, 2-3 times higher during the meat than during the other periods.

Table 5 *Changes in the urinary elimination of products of nicotinic acid and nicotinamide metabolism of rabbits on replacing the meat bread by a cabbage diet following extradietary administration of the compounds*

(Nicotinic acid or nicotinamide (200 mg) administered Figures for elimination are given as percentage of administered dose Column 2 denotes days after end of meat-bread period)

Compound administered		Metabolites eliminated			
		Rabbit 1		Rabbit 2	
		Nicotinic acid	Nicotinamide	Nicotinic acid	Nicotinamide
Total output	8th	—	58.6	—	54.6
	11th	87.7	—	87.3	—
	16th	—	56.2	—	63.3
	21st	—	73.8	—	68.1
Nicotinic acid eliminated	8th	—	10.1	—	5.2
	11th	68.5	—	73.4	—
	16th	—	18.2	—	29.0
	21st	—	20.8	—	35.9
Nicotinamide eliminated	8th	—	48.4	—	49.3
	11th	0	—	0	—
	16th	—	13.6	—	7.6
	21st	—	17.1	—	0
Trigonelline eliminated	8th	—	0	—	0
	11th	19.3	—	13.9	—
	16th	—	24.4	—	26.7
	21st	—	35.9	—	32.2

Table 6 *Effect of diet on urinary elimination of 'pseudo nicotinamide methochloride' following extradietary administration of nicotinic acid or nicotinamide to the rabbit*

(The values of 'pseudo nicotinamide methochloride' are expressed in terms of nicotinamide methochloride)

Compound administered		'Pseudo nicotinamide methochloride' elimination (mg)							
		Rabbit 1				Rabbit 2			
		Nicotinic acid		Nicotinamide		Nicotinic acid		Nicotinamide	
		100	200	100	200	100	200	100	200
Dose (mg)									
Diet									
Cabbage		0.500	0.384	0.600	0.800	0.460	0.484	0.750	0.825
Oats		0.480	0.456	0.651	0.680	0.432	0.405	0.600	0.660
Meat-bread		0.575	0.488	1.920	2.011	0.600	0.501	1.230	1.698

DISCUSSION

Two different effects of the three diets, cabbage, oats and meat-bread, were studied, i.e. that on body weight and that on nicotinic acid and nicotinamide metabolism. The caloric intake of the rabbits during the two cabbage periods was very low, but in spite of this the body weight remained constant on 63 cal/day during the first of these periods. The basal metabolic rate of the rabbits, calculated according to Evans (1945) using Meeh's (1879) formula for the surface area, was 87 cal. The apparent adequacy of this low food intake might have been due to the high temperature of the room and the static conditions of the rabbits in the limited space of the metabolism cages or to a reduced metabolic rate caused by a possible goitrogenic effect of cabbage in rabbits (Chesney, Clawson & Webster, 1928,

Webster, Clawson & Chesney, 1928, Webster & Chesney, 1928, Marine, Baumann & Cipra, 1929, Webster & Chesney, 1930, Marine, Baumann & Webster, 1930, Webster, Marine & Cipra, 1931). The far higher caloric intake of oats did not prevent a loss of body weight. Oats proved, therefore, to be inadequate for the rabbit, whilst the meat-bread mixture was much better utilized. The inadequacy of the 63 cal during the second cabbage period might be due either to an increased metabolic rate caused by the preceding oat or meat bread diet or to a depletion of the rabbit during the oat and meat periods of an essential factor which could not be supplied in sufficient amounts by the small cabbage ration.

The results of the three diets on the nicotinic acid and nicotinamide metabolism were different. Here feeding of oats after cabbage did not cause a marked

change, but feeding of the meat-bread mixture altered the metabolism essentially. No new function like amidation of nicotinic acid, characteristic of omnivorous species, was developed, but the ability to methylate was lost completely and that to deaminate was lost to a large extent when meat was fed. Both injected nicotinic acid and amide were found to be eliminated mainly unchanged in the urine. The persistence of the change in metabolism long after the return to the cabbage diet shows that this alteration is not caused directly by the actual food administered, but is due to changes, caused by the diet, in the tissues responsible for methylation and deamination. In the rat, the methylation takes place in the liver (Perlzweig, Bernheim & Bernheim, 1943, Ellinger, 1946, 1948), while the site of deamination is unknown, but might be the kidney and brain which are able to carry out the amination of nicotinic acid (Ellinger, 1946, 1948). From the fact that methylation is more quickly restored than deamination, it might be concluded that the two functions are independent of each other. The most likely explanation of the effect of the meat diet on nicotinic acid and nicotinamide metabolism is a damage of enzymic systems involved in methylation and deamination caused either by the inadequacy of the animal protein or by the lack of an essential factor in the diet.

The considerable increase in the output of 'pseudo

nicotinamide methochloride' after nicotinamide intake cannot be explained as long as the nature of this compound remains unknown. But this increased elimination may make it possible to collect sufficient amounts of this compound to study its nature.

SUMMARY

1 A change from a cabbage to an oat diet had relatively little effect on the metabolism of nicotinic acid and nicotinamide in rabbits. However, a change to a meat-bread diet suppressed the methylating mechanism completely, and the deaminating mechanism to a large extent, but no amidation of nicotinic acid as in omnivorous animals was developed. Replacing the meat-bread by a cabbage diet restored the original metabolic condition within 3 weeks. It is concluded that this altered metabolism is not due directly to the food fed, but to changes caused by the altered food intake on the tissues responsible for the metabolism.

2 The body weight was affected by the three diets in different ways. 63 cal/day were sufficient to maintain weight on the cabbage diet, while 400 cal were insufficient on the oat diet, whilst with the meat-bread diet weight was gained with 380 cal.

We wish to thank Messrs. Spratts Patent, Ltd., for a generous gift of meat meal.

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Regulation of Urinary Steroid Excretion

1 EFFECTS OF DEHYDROISOANDROSTERONE AND OF ANTERIOR PITUITARY EXTRACT ON THE PATTERN OF DAILY EXCRETION IN MAN

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In the course of investigations concerned with the steroid metabolism of mental patients, attempts were made for therapeutic purposes to influence the pattern of daily excretion of 17 ketosteroids and of cortin. The effects thereby produced throw some light on the regulatory mechanisms governing steroid metabolism. The question of how these processes are related to the mental state will be dealt with in later papers. This communication is concerned with the biochemical investigations alone, and deals with the actions of dehydroisoandrosterone and an anterior pituitary lobe extract.

EXPERIMENTAL

All the patients investigated were chronic schizophrenics in which the clinical picture was of similar form and duration. Estimations of the urinary 3(α) and 3(β) hydroxy-17 keto steroids, total oestrogen and cortin were carried out before treatment, and were later repeated at least once after treatment had commenced. One group of patients was given intramuscular dehydroisoandrosterone (50 mg/injection dissolved in 2.5 ml. ethyl oleate), and the other group was given Ambion, a pituitary anterior lobe extract containing about 50 i.u. gonadotrophic hormone and 100–200 Heye Laqueur guinea pig units thyrotrophic hormone/ml (1 ml/injection).

All urines were stored at 0° without preservative, and worked up as soon as possible after completion of collection. Creatinine estimations were performed on every separate 24 hr specimen as a test for completeness, any specimens showing gross departures from the appropriate norm of daily excretion were rejected.

17 Ketosteroid and oestrogen estimations. These were carried out on pooled 48 hr specimens. A suitable portion (20–25 l) of the urine was treated in the cold with 20 ml conc. HCl/l, then brought to the boil and boiled vigorously for 10 min. After cooling, it was transferred to a large continuous liquid liquid extractor. Extraction with benzene was carried out for a minimal period of 12 hr, and the extract was then removed and worked up according to the procedure of Callow, Callow, Emmens & Stroud (1939).

After the benzene solution containing the neutral fraction had been separated, washed and dried over Na_2SO_4 , one fifth of the volume was taken and evaporated to dryness on an oil bath at 100° (the use of a water bath was avoided to prevent contamination by water), the last traces of benzene being removed by a vacuum pump. After redissolving in 5 ml ethanol, the total 17 ketosteroids were estimated by the

Zimmermann reaction, under the conditions described by Callow, Callow & Emmens (1938). Of the remaining keto steroid solution in benzene, a portion containing 5 mg was removed, evaporated to dryness, and separated into ketonic and non ketonic fractions by a micro modification of the separation procedure employed by Callow & Callow (1938), using the Girard reagent T (13 mg Girard reagent T in 0.1 ml. glacial acetic acid were used for 5 mg ketosteroid dissolved in 0.1 ml. acetic acid). The separated ketonic fraction was further separated into α and β fractions by digitonin precipitation as described by Frame (1944). 17 Ketosteroid estimations were made on the whole ketonic fraction and on the separate α and β fractions.

The phenolic fraction of the original benzene extract was, as usual, acidified, extracted with benzene, the extract dried, evaporated and finally brought into solution in arachis oil. The total oestrogen content was assayed by the rat method, using oestradiol as standard preparation, and is expressed in arbitrary 'rat units', one rat unit being equivalent to approx 0.023 μg oestradiol. The total activity of the extracts was insufficient to permit accurate statistical evaluation.

Cortin estimations were carried out by an application of the method described by Heard, Sobel & Venning (1946), but the following modifications were introduced: (a) CHCl_3 was used as the extracting solvent, and (b) the urine was extracted continuously in the cold in an apparatus similar to that employed by Robinson & Warren (1948). The urine sample, after passing through the apparatus and collecting in a suitable vessel, was returned to the funnel and passed through the solvent again. This process was repeated until twenty such extractions had been made. By this means, the disadvantage of emulsion formation was completely avoided. Trial extractions with aqueous solutions of deoxycortico-sterone acetate confirmed that complete extraction could be effected in this way. (c) For the final estimation of the dried extract, the Hagedorn Jensen estimation, as used by Hemphill & Reiss (1947) for blood cortin, was applied. For every batch of urines taken, a water blank was carried through simultaneously, to check the purity of the various reagents.

Reagents. Benzene was purified from technical material. It was distilled once, then washed three times with water, dried over Na_2SO_4 , allowed to stand several days (at least 24 hr) over alumina with occasional shaking, and finally distilled through a 6 pear column.

Ethanol was dried by treating with Ca turnings, and then further purified by heating for 1 hr with 10 g/l semicarbazide acetate, and recovering. Acetic acid was purified by the method of Orton & Bradfield (1927).

Table 1 Daily excretion of steroids in urine of patients before and after hormone treatment

		17-ketosteroids								Oestrone
Patient and clinical diagnosis	Date	Total (mg/24 hr)	Non ketonic fraction (mg/24 hr)	Ketonic fraction (mg/24 hr)	α fraction (mg/24 hr)	β fraction (mg/24 hr)	α in ketonic (%)	β in ketonic (%)	Cortin (mg/24 hr)	(rat units/24 hr)
A Patients treated with dehydroisoandrosterone										
1 N E H, chronic schizophrenia	29 v 48	4.7	2.0	2.7	2.25	0.45	83.5	16.5	1.19	<3
	29 v 48	Commenced daily injections of 50 mg dehydroisoandrosterone								
	5 vi 48	14.7	2.1	12.6	9.5	3.1	75.3	24.7	0.84	<3
2 M G H, chronic schizophrenia	29 v 48	7.6	2.3	5.3	4.4	0.90	83.3	16.7	1.19	<3
	29 v 48	Commenced daily injections of 50 mg dehydroisoandrosterone								
	5 vi 48	25.2	4.0	21.2	14.8	6.4	69.7	30.3	0.99	—
3 P F, chronic schizophrenia	29 v 48	6.4	1.78	4.62	4.02	0.6	86.5	13.5	0.94	<3
	29 v 48	Commenced daily injections of 50 mg dehydroisoandrosterone								
	5 vi 48	34.5	9.1	25.4	24.3	1.1	95.5	4.5	1.8	—
4 L F, chronic schizophrenia	28 iv 48	8.6	2.0	6.6	5.1	1.5	77.5	22.5	1.4	<4
	29 v 48	8.3	2.5	5.8	4.5	1.3	77.2	22.8	1.38	<3
	29 v 48	Commenced daily injections of 50 mg dehydroisoandrosterone								
	5 vi 48	39.7	1.7	38.0	30.4	7.6	79.9	20.1	1.26	—
5 W C T, chronic catatonic schizophrenia	24 v 48	7.1	1.7	5.4	3.6	1.8	65.8	34.2	1.42	3
	27 v 48	Commenced daily injections of 50 mg dehydroisoandrosterone								
	1 vi 48	13.6	2.1	11.5	7.33	4.17	63.7	36.3	2.60	—
6 G R B, catatonia	25 v 48	5.0	1.55	3.45	2.53	0.92	73.3	26.7	1.5	3
	27 v 48	Commenced daily injections of 50 mg dehydroisoandrosterone								
	1 vi 48	20.8	3.0	17.8	13.7	4.04	77.3	22.7	1.61	—
7 D T, catatonia	25 v 48	5.4	3.04	2.36	2.18	0.18	92.3	7.7	0.96	4
	27 v 48	Commenced daily injections of 50 mg dehydroisoandrosterone								
	1 vi 48	15.3	3.7	11.6	7.9	3.7	67.8	32.2	0.86	3
	9 vii 48	18.7	3.5	15.0	12.1	2.9	81.0	19.0	2.31	12
	15 vii 48	21.8	3.0	18.8	15.5	3.3	82.4	17.6	1.07	4
	11 viii 48	Injections stopped								
8 R C D, chronic schizophrenia	24 ix 48	6.8	1.6	5.2	4.56	0.64	87.6	12.4	—	—
	25 v 48	10.4	3.25	7.15	5.55	1.60	77.4	22.6	0.82	4
	27 v 48	Commenced daily injections of 50 mg dehydroisoandrosterone								
	1 vi 48	27.3	5.1	22.2	14.7	7.5	66.2	33.8	1.93	12
	6 vii 48	32.8	1.6	31.2	20.6	10.6	66.0	34.0	3.6	8
	15 vii 48	39.3	8.1	31.2	20.7	10.5	66.2	33.8	2.1	4
	11 viii 48	Injections stopped								
	24 viii 48	9.3	3.1	6.2	5.9	0.30	95.8	4.2	1.54	—
B Patients treated with Ambinon										
9 J E D, simple schizophrenia	26 iv 48	11.4	2.0	9.4	8.08	1.32	86.0	14.0	1.53	3
	1 vii 48	6.9	0.7	6.2	5.37	0.83	86.6	13.4	1.60	—
	6 vii 48	Commenced daily injections of Ambinon								
	10 vii 48	11.7	2.0	9.7	9.22	0.48	95.1	4.9	1.32	—
	17 vii 48	5.9	1.6	4.3	3.98	0.32	92.5	7.5	—	24
	22 vii 48	Injections stopped								
10 E K, paranoid schizophrenia	26 viii 48	7.5	2.3	5.2	4.8	0.40	92.3	7.7	—	<3
	23 vi 48	13.7	3.4	10.3	7.3	3.0	71.0	29.0	1.63	—
	29 vi 48	13.5	2.8	10.7	8.2	2.5	76.8	23.2	1.59	8
	6 vii 48	Commenced daily injections of Ambinon								
	10 vii 48	19.1	4.2	14.9	11.8	3.1	79.0	21.0	1.46	24
	17 vii 48	17.8	2.6	15.2	12.1	3.1	79.4	20.6	—	24
	16 viii 48	12.9	3.1	9.8	6.5	3.3	66.0	34.0	—	—
11 J A L, chronic schizophrenia	22 viii 48	Injections stopped								
	27 viii 48	15.2	4.3	10.9	8.4	2.5	76.5	23.5	—	6
	5 v 48	5.1	0	5.1	3.6	1.5	69.9	30.1	0.94	4
	12 v 48	6.3	2.6	3.7	3.38	0.31	91.7	8.3	0.83	—
	12 vi 48	7.6	3.2	4.4	3.91	0.49	88.8	11.2	1.03	6
	14 vi 48	8.9	4.0	4.9	4.74	0.16	96.7	3.3	0.93	—
	27 vi 48	Commenced daily injections of Ambinon								
12 A J A, chronic schizophrenia	6 vii 48	4.1	1.3	2.8	2.64	0.16	94.2	5.8	1.15	4
	15 vii 48	2.4	—	—	—	—	—	—	0.44	4
	29 vi 48	16.2	4.3	11.9	11.03	0.87	92.7	7.3	2.00	no response
	6 vii 48	9.0	2.9	6.1	5.7	0.40	93.7	6.3	1.54	>6
	10 vii 48	5.1	1.3	3.8	3.55	0.25	93.4	6.6	—	—

RESULTS

The results for the whole series of estimations are recorded in Table 1. It will be seen that the administration of the 3(β)-hydroxy-17-ketosteroid dehydroisoandrosterone caused a considerable rise in the excretion of the α fraction of 17-ketosteroids in all of the eight patients receiving this preparation. In every case, except one, the β fraction was also increased, but not to the same extent as the α fraction. Cortin was increased in three cases (nos 3, 7 and 8) and total oestrogen was increased in two cases (nos 7 and 8).

Ambinon caused a reduction in the α fraction of 17-ketosteroids, accompanied in most cases by a definite rise in total oestrogen. No marked changes in cortin excretion were seen in these cases.

DISCUSSION

The remarkable increase in the excretion of α fraction of 17-ketosteroids following administration of dehydroisoandrosterone may be compared with similar phenomena reported by other workers. Dorfman, Wise & Shipley (1948) demonstrated increased 3(α) hydroxy-17-ketosteroid excretion in one patient after the injection of a 3(β)-hydroxy-17-ketosteroid (isoandrosterone). Mason & Kepler (1947) also studied the ketosteroid excretion of two patients suffering from Addison's disease who received over 1000 mg of dehydroisoandrosterone during 12 days, administered while the adrenal insufficiency was controlled by treatment with deoxycorticosterone and sodium chloride. Subsequent examination of the urine revealed no dehydroisoandrosterone, but only androsterone, aetiocholanolone, and other 3(α) hydroxy-ketosteroids, which accounted for 43 and 19 % respectively of the injected hormone. These authors assumed that dehydroisoandrosterone is the precursor of the α steroids mentioned, under normal conditions. It is, however, debatable whether this increase of α steroid excretion is due to a direct chemical conversion of the β steroid, as Dorfman *et al* (1948) and Mason & Kepler (1947) assume. One has to account for the fact that the 50 mg dehydroisoandrosterone injected daily resulted in increases of total ketonic fraction in the range of 12–64 % of the injected dose only in the first days after commencing the dosage. Moreover, the simultaneous increase of cortin excretion in three cases and of total oestrogen in two cases are hardly to be accounted for by a purely chemical mechanism. Little is known as yet about the physiological action of dehydroisoandrosterone on the gonads or adrenals, but it is feasible that a stimulation of the adrenal cortex or gonadal elements by this substance may have taken place, a more detailed examination of this possibility may well prove to be of considerable clinical importance.

The remarkable increase in cortin excretion in one

case, and the slight but significant rise in two cases might point to some stimulatory influence on the production of C_{21} steroids. It is known that injection of deoxycorticosterone brings about a reduction in adrenal cortical function, with decreased hormone production. Since dehydroisoandrosterone is produced for the most part in the adrenal cortex, it is possible that in some patients the endogenous production is reduced when the compound is administered, thereby increasing the availability of the natural precursor for the production of C_{21} steroids. It is possible that competition between production of dehydroisoandrosterone and of cortin may exist, a regular inverse relationship between cortin excretion and β -steroid excretion has in fact been observed in these laboratories in a patient showing cyclic changes of activity and depression (full details of this work will be published later). During depression the β steroid excretion was increased, and the cortin excretion decreased, and the reverse tendency was shown during normal and maniacal phases. In this connexion it is of some interest to note that the patient who showed the greatest rise in cortin excretion after dehydroisoandrosterone was the only one who showed complete mental improvement, with subsequent discharge from hospital.

Regarding the decreased α -steroid excretion (which was preceded in two cases by an increase) brought about by Ambinon, it may be assumed that this is due to the gonadotrophic fraction contained in this preparation. Previous work (Carreyett, Golla & Reiss, 1945) has shown that gonadotrophic hormone prepared from pregnant mare serum decreases the total ketosteroid output in patients, and depletes the lipid content of rat adrenals, after several days' treatment. It is thus evident that dehydroisoandrosterone and Ambinon produce opposite effects on the excretion of 3(α)-hydroxy-17-ketosteroids. It remains to be seen, however, to what extent they are antagonistic in the strict sense.

SUMMARY

1 Administration of dehydroisoandrosterone produced in several mental patients a marked rise in the excretion of the 3(α)-hydroxy-17-ketosteroids. Increases of cortin and of oestrogens were noted in a few cases.

2 The anterior pituitary lobe extract, Ambinon, produced a net fall in the excretion of the 3(α) hydroxy 17-ketosteroids, usually accompanied by a rise in total oestrogen excretion.

3 Possible mechanisms for the changes described above are discussed.

The authors wish to acknowledge their indebtedness to Organon Laboratories Ltd., for supplies of dehydroisoandrosterone and Ambinon preparations, to Messrs W G Warren, D J Dell and Miss B Binham for technical assistance, and to Mr F Knight for valuable co operation in the collection of urine specimens.

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The Use of the Waring Blender in Biochemical Work

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In recent years the Waring blender has found widespread application in biochemical work, owing to the ease and speed with which it disintegrates all sorts of heterogeneous materials into uniform suspensions. It is surprising that the literature contains no reference, so far as the authors are aware, to possible harmful effects of treatment in the Waring blender on the properties of biological material. Several workers seem to have noticed that the intense aeration occurring in operating the blender causes oxidation of reducing substances. This can be gathered from the fact that they use the blender *in vacuo* or in an atmosphere of inert gas. However, no definite information on this point seems to have been published. A further possibility of damage to biological material might be anticipated, viz denaturation of proteins and hence inactivation on enzyme systems through intensive agitation.

While carrying out experiments on the reducing matter and oxidizing enzymes of wheat germ and other mill streams, the authors found that even short treatment in the Waring blender greatly affected these systems. The present paper describes these findings which were first reported at the New Zealand Science Congress held in Wellington in 1947. In the meantime Quinlan-Watson & Dewey (1948) reported inactivation of cytochrome c oxidase caused by treating animal tissue in the Waring blender. The main work on oxidizing enzymes of wheat will be the subject of separate papers.

EXPERIMENTAL

Quantitative evidence for the effect of the Waring blender on suspensions of various mill streams comes from the following experiments

Effects of the Waring blender on reducing matter

Suspensions of wheat germ (e.g. 1 part germ and 11 parts of water) were made (a) by grinding with sand and water in a mortar, (b) by treating in the Waring blender, and (c) by boiling for 2 min. In the blender, the temperature of a 250 g suspension rose by 18° within 5 min owing to the generation of heat by friction. To avoid injurious increases of temperature on treating the suspensions for more than 5 min they were cooled after 5 min stirring, or ice water was used for making them.

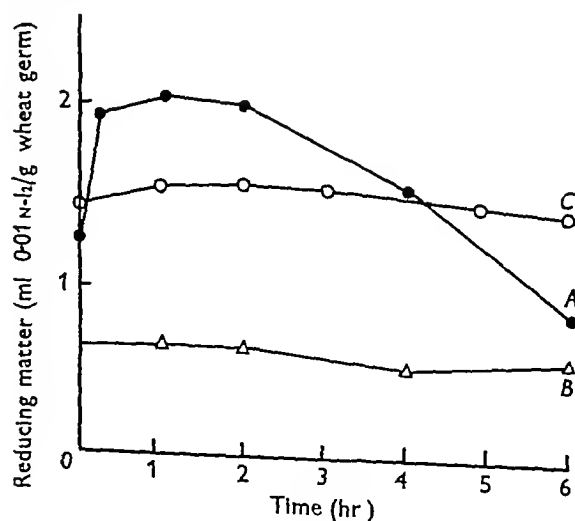


Fig 1 Changes of reducing matter in wheat germ suspensions obtained A, by grinding with sand, B, by 5 min treatment in the Waring blender, C, by boiling and allowing to stand at 40° for several hours

The suspensions were then kept in a water bath at 40°, and samples for determination of reducing matter were withdrawn at various intervals. The results were expressed as ml 0.01 N-I₂ solution/g of germ. The changes of reducing matter with time are shown in Fig 1. These graphs in the

first place demonstrate that treatment in the Waring blender caused oxidation of the bulk of the reducing matter present in wheat germ

Fig 1 (curve A) shows that in the sand ground suspension there is an initial increase and a subsequent decrease of reducing matter. Both are due to enzyme action they do not occur in boiled suspensions (curve C). The increase is probably the effect of a protease liberating thiol groups, the decrease that of an oxidizing enzyme mechanism. Curve B (suspension treated in Waring blender) closely resembles curve C (heated suspension) except that it is on a lower level. It seems to indicate that treatment in the Waring blender destroyed enzyme activities responsible for the liberation and oxidation of reducing matter (shown by curve A).

Sullivan, Howe & Schmalz (1936) found that wheat germ contains GSH which causes the severe damage, well known to cereal chemists, accompanying the inclusion of wheat germ in doughs. Hullett & Stern (1941) and Smith & Geddes (1942) showed that GSH could be eliminated from wheat germ by fermentation, and that such pre-fermented wheat germ had no adverse effect on the doughs. The elimination of GSH by fermentation, indicated by negative reaction with sodium nitroprusside, takes several hours, treatment in the Waring blender achieved the same result within 5 min. A wheat germ suspension stirred in the Waring blender for 5 min gave a negative reaction with nitroprusside and was quite harmless when included in the dough. This rapid oxidation of the thiol groups is due to an enzyme mechanism which requires the presence of O_2 . No comparable oxidation takes place in a boiled germ suspension treated in the blender.

Effect of the Waring blender on dehydrogenase activity

The dehydrogenase activity of wheat germ extract made from 1 part of germ and 5 parts of water, measured by the Thunberg technique at 30° , dropped from a decoloration time of 20 min for the untreated sample to one above 4 hr for a sample treated in the Waring blender for 2 min.

Effect of the Waring blender on ascorbic acid oxidase activity

The discovery of ascorbic acid oxidase in wheat flour by Melville & Shattock (1938) was confirmed by Sandstedt & Hites (1945) who also observed that ascorbic acid oxidase was responsible for the oxidation of the thiol groups present in wheat flours.

In experiments on the determination of ascorbic acid oxidase activity in various mill streams, the following technique was used. Mill stream (10 g) was suspended in 170 ml of 0.2M phosphate buffer of pH 6.5 and digested at 30° for 0.5 hr. The suspension was shaken every 10 min. Ascorbic acid solution (20 ml, containing 20 mg ascorbic acid and 20 mg HPO_3) was added so as to make a total of 200 g of suspension. Immediately after adding the ascorbic acid a sample of 20 g was removed for determination of reducing substances. The remainder of the suspension, still kept at 30° , was aerated with a strong current of air which was stopped after exactly 20 min. Previous experiments had shown that the oxidation over the initial 20 min was linear.

A vigorous air stream is necessary to ensure good replication of results. After 20 min aeration a second sample of 20 g was removed from the reaction mixture. Both samples, immediately after being taken, were deproteinized with 2 ml of 10% (v/v) H_2SO_4 and 2 ml of 12% sodium tungstate, made up to 200 g and centrifuged at 2000 rev/min. Previous

experiments had shown that if mill streams were deproteinized under these conditions ascorbic acid could be recovered quantitatively, and the centrifugates were clearer than those obtained with HPO_3 .

To 50 ml of the supernatant (corresponding to 0.25 g of flour) were added 5 ml of 0.002N I_2 solution and the excess was back-titrated with 0.002N- $Na_2S_2O_3$. The difference between the readings before and after aeration, expressed in ml 0.01N I_2 solution/g flour, was taken as a measure of ascorbic acid oxidase activity of the flour suspension. It was found that all readings were replicable, with a standard of ± 0.05 ml 0.01N- I_2 solution (corresponding to roughly 2% of the initial reading), and that the initial reading corresponded to the sum of added ascorbic acid and reducing matter present in the flour.

When wheat-flour suspension was treated in the Waring blender for 2 min, and then tested for ascorbic acid oxidase activity as described above, the results differed greatly from those obtained with a control prepared by 0.5 hr digestion at 30° (Table 1).

Table 1 *Oxidation of ascorbic acid in untreated and Waring blender-treated flour suspensions*

(Reducing substances in 50 ml of supernatant from flour suspensions (corresponding to 0.25 g flour + 0.5 mg ascorbic acid) were determined before and after aeration. Results are expressed as ml 0.01N- I_2 /g flour. The difference between results before and after aeration is a measure of the oxidation of ascorbic acid occurring during aeration.)

Duplicate determinations were carried out on a control suspension prepared by digestion for 30 min and on a suspension prepared by 2 min treatment in the Waring blender.)

	Control suspension prepared by digestion (ml 0.01N I_2 /g)	Suspension prepared by 2 min treatment in Waring blender (ml 0.01N I_2 /g)
Initial reading	2.41, 2.37	2.15, 2.00
Final reading	1.95, 1.93	1.24, 0.76
Difference	0.46, 0.44	0.91, 1.24

A comparison of columns 2 and 3 shows the following effects of treatment in the Waring blender: (1) duplicate determinations give widely varying results, (2) the initial readings are lower than 2.27 ml, which is the value corresponding to the added ascorbic acid, (3) differences between initial and final readings are much larger than in the control. These three effects of treatment in the Waring blender correspond exactly to what happens when ascorbic acid oxidase in a flour suspension is destroyed by high temperature or pH. Under these conditions the enzyme mechanism breaks down and is superseded by aerial oxidation. It seems very probable, therefore, that treatment in the Waring blender destroyed ascorbic acid oxidase.

Other effects of the Waring blender

Fresh wheat-germ suspensions have a bright yellow colour. On oxidation they assume a greyish white hue, and later, particularly on acidification, turn a deep purple on the surface and wherever else they are in contact with air as on the walls of included air bubbles. Suspensions treated in the Waring blender never proceeded beyond the greyish white stage. It was thought that tyrosinase or peroxidase might be responsible for the darkening effect. Tyrosinase determina-

tions by the chronometric method of Miller & Dawson (1941), showed that wheat germ contains no measurable amount of tyrosinase. Peroxidase, on the other hand, was present and qualitative reactions with guaiac and with benzidine, remained positive even after wheat-germ suspensions had undergone 13 min treatment in the Waring blender. This behaviour of wheat peroxidase is in keeping with the high resistance of peroxidases from various sources to conditions which are injurious to other enzymes, e.g. high temperature. The fact that wheat peroxidase survives treatment in the Waring blender would seem to indicate that the darkening effect is not due to peroxidase action since failure to darken after treatment is not connected with destruction of peroxidase. It is to be assumed, however, that peroxidase is only one member of a more complex enzyme mechanism which may provide H_2O_2 necessary for peroxidase action. For this reason failure to darken might be due to the breakdown of some other part of this enzyme mechanism. An investigation of the darkening effect is in progress.

DISCUSSION

Potter & Elvehjem (1936) described a device for grinding tissue material, and reported that enzyme inactivation occurred on dilution of the ground material. According to their explanation, dilution causes the coenzyme concentration to drop below the level necessary to maintain enzyme activity. This explanation does not apply to the enzyme inactivations reported in the present paper. While

Potter & Elvehjem compare suspensions of different concentrations the present paper deals with suspensions of equal concentration, but obtained by different means, viz. by grinding with sand in one case and by treatment in the Waring blender in the other.

The Waring blender, with its speed of about 10,000 rev/min is far more likely to cause protein denaturation than the grinder used by Potter & Elvehjem (1936), with its much lower speed. The present authors suggest that, while in their experiments oxidation of activating thiol groups may have been responsible for the inactivation of certain enzyme systems, the main damage caused by the Waring blender was perhaps enzyme destruction due to protein denaturation.

The experiences reported above make it justifiable to sound a note of caution with regard to the Waring blender and similar homogenizers, and to recommend restricting their use to applications which do not involve inactivation of enzymes or in which such inactivation does not matter.

SUMMARY

Treatment of mill stream suspensions in the Waring blender caused oxidation of thiol groups and inactivation of enzyme systems.

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A Mercury Gasometer for the Preparation of Gas Mixtures

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The need for a means of preparing gas mixtures containing accurately known proportions of carbon dioxide arose during manometric studies of tissue metabolism. The measurement of glycolysis in terms of CO_2 liberated from a bicarbonate buffer requires an atmosphere containing a small proportion of CO_2 , and cylinders of nitrogen and oxygen containing 5% of CO_2 are commercially available. When, however, different concentrations of CO_2 or mixtures of CO_2 with more than one other gas are required, one of the following methods must be employed.

- (1) A continuous flow method in which flowmeters measure the rate of delivery of the individual gases.
- (2) Collection of the gases, in a graduated Mariotte bottle or other container, over a saturated salt solution in which gases are generally less soluble.
- (3) Preparation of the gas mixture in a pressure vessel fitted with a gauge.
- (4) Collection of the gases in a graduated vessel over mercury.

The flowmeter method is unreliable since the proportions in the mixture will vary widely with changes both in individual gas pressures and in resistance to

APPARATUS

The mercury gasometer (Fig 1) consists of two identical graduated Pyrex glass mixing cylinders of 1500 ml capacity each connected by reinforced rubber tubing, through a 2 way glass tap, to two arms of a glass T piece. The third arm of the T piece is connected to a glass reservoir of just over 1500 ml capacity. At the top of each cylinder is a 3 way tail pattern tap and the upper half of the cylinder, which is 4.3 cm in external diameter, is graduated from 0 (at the tap) to 300 ml at intervals of 15 ml. The lower half is somewhat wider (7.5 cm diameter) and 100 ml graduations are continued down this section to the 1500 ml mark which is at the upper end of the neck to which the reinforced rubber tubing is attached. The distance between the 1500 ml graduation and the top is 63 cm. The neck rests within a fibre ring which fits into a hole in the lower steel plate of the cylinder carrier. The reservoir is similarly supported in a fibre ring (FR).

The framework within which the glass parts are supported was manufactured in mild steel and consists of a base plate (BP), 30.5 × 40.5 × 1.0 cm, to which are bolted five perpendicular 20 mm diameter rods (R_1, R_2). The upper ends of these rods are bolted to a top plate (TP) which is out away so as to permit the upper parts of the glass vessels to pass through. The distance between top and base plates is 107 cm. To the top plate are bolted two pulley supports (PS) each carrying a pair of grooved pulleys. Over each pair of pulleys passes a stranded steel cable (2 mm in diameter), one end of which is attached to a steel carrier in which glass parts are supported while the other end winds around an 8 cm diameter flanged pulley (Pu). These flanged pulleys are mounted on an extension to the pinion, or driven, shaft of two tennis net winders (Te) bolted to the base plate. In order to reduce the strain on the winder bearings, the shaft extensions are supported in extra bearings (Be). The two glass mixing cylinders are supported on the fibre rings within a carrier consisting of two horizontal, 5 mm thick, steel plates (P) which are separated by three 26 cm lengths of steel tube about 22 mm internal diameter. The rods R_1 pass through these tubes and act as guides when the carrier is raised or lowered. In the upper plate are two holes to take the cylinders and in the lower plate are two smaller holes into which fit the fibre rings. The steel cable passes down through a small hole in the upper plate and is retained by means of a terminal blob of hard solder. The other end of the cable is retained in the same way on the flanged pulley. The carrier for the reservoir is similarly built up on two 23 cm lengths of steel tube moving along the rods R_2 . It incorporates a stirrup above the upper plate consisting of two 13 cm threaded rods to which is screwed by means of milled nuts a horizontal \perp section bar (Ba). This bar, to which the steel cable is attached, has a hole at one end for the threaded rod and a slot at the other so that it may be swung aside when it is necessary to add mercury or to remove the reservoir. Each of the five steel rods (R_1, R_2) carries two collars (Co) with set screws which are fixed so as to limit the travel of the carriers.

If these are raised too far when rubber tubing is attached to the tail pattern taps there is a risk of breakage should the tubing become strained as it passes through the top plate. On the other hand, if the carriers are allowed to go too low a strain is thrown on the mercury containing rubber tubes attached to the lower outlets of the glass vessels.

The connexions between the three vessels are made with canvas interlined rubber tubing of 10 mm internal diameter. This gives a tight fit over the necks of the glass vessels which are drawn out to 10 mm diameter with a bulge to 11 mm. The glass T-piece (T) and pressure taps (PT) are screwed down by means of saddles to strips of wood bolted to the base plate. These strips are thick enough (2.5 cm) to accommodate, when suitably drilled, the projecting spring loaded barrels of the taps of the type used in the Van Slyke apparatus. By means of these taps it is possible to isolate one or both of the mixing cylinders from the reservoir. This layout was adopted since it appeared impracticable to have two reservoirs and a double charge of mercury. The present design requires 22 kg mercury.

The rubber to glass joints are bound with insulating tape and copper wire, this procedure eliminates the risk of splits in the rubber tubing. Only after 12 years was it found necessary to replace the tubing on account of its decreasing pliability. The only other 'servicing' that the apparatus requires is lubrication of the bearings and of the steel cables which should be occasionally rubbed with soft paraffin ('Vaseline').

The gasometer is mounted on a wheeled angle iron trolley by means of bolts passing through the base plate and through a similar plate, 65 cm from the ground, which forms the top of the trolley. A drain plug (DP) facilitates the collection of spilled mercury.

USES OF THE GASOMETER

In addition to its value for the preparation of mixtures of water soluble gases, the apparatus serves well for making up gas mixtures whenever a higher accuracy is required than can be obtained over water where a certain gas exchange between the two phases is inevitable. It is thus of great advantage in preparing, by serial dilutions, a mixture containing a very small proportion of one constituent (e.g. CO in O₂ for haemoglobin studies). The gasometer is also useful for the storage of highly purified gases or of gases, such as nitric oxide, which react with water or with oxygen.

SUMMARY

A description is given of a mercury gasometer in which two gas mixtures of up to 1500 ml in volume can be accurately made up.

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 Entries marked with a section mark (§) refer to contributions to Symposia
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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 272nd Meeting of the *Biochemical Society* was held at the British Postgraduate Medical School, Ducane Road, Hammersmith, London, W 12, on Saturday, 22 January 1949, at 11 a m, when the following papers were read

COMMUNICATIONS

The Micro-estimation of Benzoic and Hippuric Acids in Biological Material By F DICKENS and JOAN T PEARSON (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W 1*)

Existing methods for the micro-estimation of benzoic acid have not been found satisfactory When hot nitration is employed as a preliminary stage (Waelsch & Klepetar, 1935) we find that a complex mixture of nitro-compounds results, of which 2,5-dinitrobenzoic acid appears to be mainly responsible for the colour development on treatment with hydroxylamine This product has been isolated and constitutes only c 7% of the benzoic acid sample

By the use of cold nitration, *m* nitrobenzoic acid is made to constitute the main product, and colour development from this stage follows well-known lines

Neutralized samples containing benzoic or hippuric acid are evaporated to dryness and the residue is nitrated with 10% KNO_3 in cold conc H_2SO_4 The

nitration product is extracted with isoamyl alcohol and then into alkali, reduced in acid solution with titanous chloride to *m*-aminobenzoic acid, which is diazotized with NaNO_2 and the product coupled with *N*(1-naphthyl)-ethylene diamine (Eckert, 1943) The magenta colour is estimated by the Hilger absorptiometer The method is suitable for the estimation of samples containing up to 10 mg benzoic acid Linear relationships of quantity of benzoic acid to intensity of colour have been shown to hold over the range 0.05–10 mg The same factor applies both to benzoic and hippuric acids The method is particularly suited to the estimation of benzoic acid formation by tissues, the preliminary extraction being made into 60% ethanol Phenolic and other interfering substances if present must be removed before nitration

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The Formation in Animal Tissues of Benzoic Acid from Possible Hydroaromatic Precursors

By C T BEER, F DICKENS and JOAN T PEARSON (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W 1*)

The conversion of cyclohexane carboxylic acid to benzoic (or hippuric) acid in the rabbit has been shown to be an aerobic oxidation occurring mainly in the liver tissue and to a lesser extent in the kidney (Dickens, 1947)

Nothing is known of the mechanism of this reaction though it presumably occurs in successive stages, e.g. by the stepwise introduction of double bonds The latter might arise either by direct dehydrogenation or by the preliminary introduction of a group, e.g. —OH, followed by its subsequent elimination together with a hydrogen atom One example of the biological introduction of an —OH group into the cyclohexyl ring has recently been mentioned by Fieser (1947)

In order to test these hypotheses, we have studied the effect of several tetrahydro- and monohydroxy-hexahydrobenzoic acids on the aerobic metabolism of rabbit-liver slices The latter were incubated at 37° in the Warburg apparatus with phosphate-Ringer solution containing 0.05–0.001M-sodium salts of these acids After 4 hr the benzoic acid formed was determined by the method of Dickens & Pearson (1948)

No significant amounts of benzoic (or hippuric) acid were obtained from *trans*-4-hydroxy-, *cis*-4-hydroxy-, *cis*-3-hydroxy-, or 1-hydroxy-cyclohexane carboxylic acids The two 4-hydroxy compounds tended to increase slightly, and the remaining compounds to diminish, the respiration rate A mixture

of Δ^1 and Δ^2 -tetrahydrobenzoic acids containing 85 % of Δ^1 , had no appreciable effect on respiration rate and gave little benzoic acid either in liver or kidney slices. No benzoic acid was obtained from cyclohexanone-2-carboxylic acid.

In the intact rabbit injection of the same mixed Δ^1 - Δ^2 acids and of pure Δ^2 tetrahydrobenzoic acid (as Na salts) was followed by urinary excretion in 24 hr of respectively 40 and 15 % as benzoic acid. The behaviour of the Δ^3 compound is being studied. Neither *cis* 4-hydroxy- nor 1-hydroxy cyclohexane carboxylic acid yielded benzoic acid in the rabbit.

In man, quinic acid (1 3 4 5 tetrahydroxy hexa hydrobenzoic acid) is converted into hippuric acid (Lautemann, 1863, Quick, 1931). We have confirmed this by isolation from the urine of c. 70 % of doses of 6 g quinic acid as pure hippuric acid. Unfortunately a similar change was not found in the rabbit, guinea pig, or rat, nor could the aromatization of quinic acid be detected in slices of rabbit or sheep liver, although the rabbit and sheep have been reported to form benzoic acid from ingested quinic acid (Vasiliu, Timosencu, Zaimov & Coteleu, 1940), since these authors report only 10 % conversion of quinic acid in man their indirect methods are open to doubt.

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Ancillary Apparatus for Chromatography By S S RANDALL and A J P MARTIN (*Boots Pure Drug Company Ltd, Nottingham*)

When colourless substances are chromatographed numerous portions of the effluent have to be tested and suitably grouped if optimum separation is to be obtained. Moore & Stein (1948) have described a photoelectrically operated fraction cutter which can collect 80 equal fractions. Apparatus described here is mechanical and very simple. The effluent runs from the column into a siphon suspended from a balance beam by a twisted cord, which is above the centre of a circular test-tube rack. The siphon has a long arm delivering directly into 114 test-tubes. On the arm is a simple escapement engaging with pins opposite each tube. The filling and emptying of the siphon causes the balance beam to rock, the vertical movement of the siphon operates the escapement, and the siphon delivers once into each tube in turn, the twisted cord providing power for the rotation of the siphon. The design of the siphon is critical and different siphons are required for H_2O , Et_2O , $CHCl_3$,

etc. Delivered samples of alkali showed a range of ± 3 % on titration.

Ions may be detected in the effluent from a chromatogram, or in the stationary phase of a partition chromatogram using suitably placed platinum electrodes, by changes in conductivity. 1000 cycle a.c. is fed to a bridge containing the electrodes and the slide wire of a Cambridge recording galvanometer. The out-of-balance current, after amplification, is fed to a phase-distinguishing bridge, containing a pair of matched diodes, which delivers a d.c. output (whose sign depends upon the direction of the error of setting of the first bridge) to the recording galvanometer which automatically corrects the error. The movements of the siphon of the fraction cutter are recorded on the same chart as the conductivity.

Records obtained with penicillin, streptomycin, and other substances were shown.

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Effect of Body Exposure to X-rays upon Bone Marrow By C LUTWAK-MANN and F W GUNZ
(*Biochemical Laboratory and Department of Radiotherapeutics, University of Cambridge*)

In the course of studies on certain aspects of bone-marrow metabolism (Lutwak-Mann, 1947*a, b*) an investigation was carried out on the effect of total body exposure to X-rays upon the bone marrow of rats and rabbits. A single dose of 1000 r was given to rabbits (littermates, 1.8–3 kg) and 500 r to rats (inbred stock, 200 g). The period of observation was 2 weeks, there was some loss of appetite and slight fall of weight, in rats occasionally mild diarrhoea. Changes were observed in the appearance of the bone marrow. Already 6 hr after irradiation rat bone marrow was semi-fluid, after 1–2 days there was considerable admixture of blood whereas the haemoglobin content of marrow in untreated rats was comparable to blood diluted 1:15, in irradiated rats at this stage it corresponded to blood diluted 1:2 or 1:4, specimens 6–8 days later were 'fatty' but of a thicker consistency and a brownish pink colour, with a haemoglobin content like blood diluted 1:9. Rabbit bone marrow was relatively little altered until 3–4 days after irradiation, when it frequently appeared oedematous and gelatinous, sometimes, 10–14 days later, it still remained oedematous.

With the above doses bone-marrow glycolysis and

respiration in rats and rabbits was little affected at 6 and 24 hr after exposure, at 3–6 days it was very low, there was partial recovery in glycolysis of rats at 8 days, and in respiration at 10 days, after irradiation. In rabbits low glycolysis and respiration persisted sometimes as late as 11 and 13 days, following irradiation.

Nucleic acid P showed a noticeable decrease in rat bone marrow 6 hr after irradiation. Lowest values for nucleic acid P in both rat and rabbit bone marrow were found between the second and fourth day, but even 14 days after exposure it was still considerably less than in controls. ATP in rabbit bone marrow fell a few days after irradiation and failed to return to normal 14 days later.

Histologically, sections of rabbit bone marrow taken 24 hr after exposure showed some mitotic abnormalities. At 3 days there was evidence of severe damage, such as diminution in cellularity and almost complete absence of mitoses. Specimens taken 6 days after irradiation as well as at later stages showed distinct signs of recovery, although the metabolic measurements frequently remained below those found in untreated rabbits.

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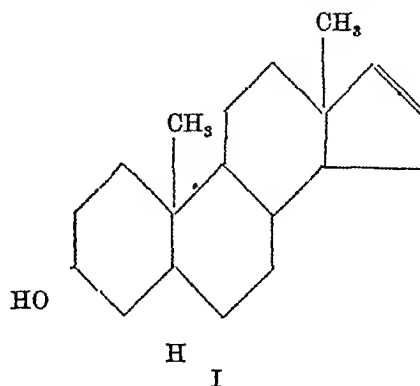
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Investigations on Urinary 'Pregnanediol-like Glucuronide' (PLG) The Isolation of Δ^{16} -androst-3(α)-ol from Normal Human Male Urine By B W L BROOKSBANK and G A D HASLEWOOD (*Physiology Department, Guy's Hospital Medical School, London, S E 1*)

A method has recently been described (Bisset, Brooksbank & Haslewood, 1948) for the estimation of sodium pregnanediol glucuronide (NaPG), based upon entrainment (as the barium salt), on a precipitate of barium phosphate and measurement of the colour produced by reaction of the glucuronic acid with naphthoresorcimol. This method, when applied to urines, gave results which clearly indicated that in addition to pregnanediol glucuronide other glucuronides of similar solubility were being estimated.

Having found that the content of normal male urine in this 'pregnanediol-like glucuronide' (PLG) was considerable (of the order of 5–7 mg/24 hr specimen), we have extracted PLG from this source on a large scale. The material was extracted with *n*-butanol and entrained on barium phosphate as in the quantitative method, it was recovered from the precipitate by dissolving this in HCl and re-

extracting with *n*-butanol. Further purification of the crude PLG obtained in this way was attained by chromatographic adsorption on alumina.



Acid hydrolysis of the glucuronide was unsatisfactory. With the collaboration of Dr M Barber, we therefore used a strain of *Staph albus*. Certain

strains of this micro-organism would hydrolyze NaPG, whilst some other bacteria would not (Barber, Brooksbank & Haslewood, 1948). Good recovery of free pregnanediol was obtained after incubation in 'Lablemco' broth of 100 mg of 'NaPG' (Venning) with *Staph albus*.

The ether soluble material from incubation of a similar quantity of PLG (assaying at 76% purity, calculated as pure anhydrous NaPG) was purified by chromatographic adsorption on alumina Δ^{16} -Androsten-3(α) ol (I), which smells strongly of musk, was obtained in the first (light petroleum) eluates. It was freed from hydrocarbon like impurities with methanol, crystallized from aqueous acetone, sublimed *in vacuo* and finally crystallized from light

petroleum. The final m.p. was 143–143.5°, mixed m.p. with authentic material (kindly supplied by Dr Prelog of Zurich), 142–3° (Found (Weiler) C, 83.00, H, 11.26. Calculated for $C_{19}H_{30}O$ C, 83.20, H, 10.95%).

Δ^{16} -Androsten-3(α)-ol has previously been isolated from swine testes, together with the C_3 epimer (Prelog & Ruzicka, 1944). We consider that its formation by bacterial action in our experiments is extremely unlikely, particularly as the presence of the β -compound in our material has been indicated by digitonin precipitation.

The other constituents of hydrolyzed PLG are being investigated.

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Urinary Cholesterol in Cancer 1 Chemical State of Urinary Cholesterol and Methods of Estimation By M. BURCHELL and N. F. MACLAGAN (*Department of Chemical Pathology, Westminster Medical School, London*)

Reasons are given for doubting the specificity of certain previous extraction and precipitation methods of estimating urinary cholesterol. A new method of estimation depending on aluminium tungstate precipitation has been developed which is suitable for normal supernatant urine. Adsorption on alumina was successfully employed in the estimation of the cholesterol in deproteinized urine but did not recover the whole of the cholesterol from normal untreated urine.

Experiments involving adsorption, aluminium tungstate precipitation and dialysis indicate an association between the supernatant urine cholesterol and a proteose fraction. Much or all of the urinary cholesterol may at times be found in the urinary deposit, or associated with heat coagulable protein when present.

Urinary Cholesterol in Cancer 2 Urinary Cholesterol Excretion in Cancer and Control Subjects By M. BURCHELL, J. H. O. EARLE and N. F. MACLAGAN (*Department of Chemical Pathology, Westminster Medical School, London*)

Values are given for the 12-hourly excretion of urinary cholesterol in 43 control subjects and in 79 patients suffering from various forms of cancer. The cholesterol in the urine deposit (UD) normally varies from 0 to 0.05 mg/12 hr in men, and from 0 to 0.42 mg in women. The supernatant urine (SU) cholesterol varies from 0 to 0.5 mg/12 hr with no sex difference. Albuminuria if present increases the SU cholesterol, haematuria or pyuria increases the UD cholesterol.

In 54 cases of cancer without albuminuria the SU cholesterol was above normal in five, three of these cases having only slightly raised values. In 25 cases of cancer without haematuria or pyuria the UD cholesterol was within normal limits. In 57 cases of cancer without albuminuria, the SU proteose was above normal in four cases.

Estimation of the Anti-pernicious Anaemia Factor By W F J CUTHBERTSON (*Research Division, Glaxo Laboratories Ltd, Greenford, Middlesex*)

A growth factor for *Lactobacillus lactis* Dorner ATCC 8000 has been shown by Shorb (1947) to be present in highly refined liver extracts. The amounts present were, moreover, stated to be proportional to the anti-pernicious anaemia activity of these extracts. We could not develop a satisfactory assay procedure on the basis of the meagre information published by Shorb, who herself states (Shorb, 1948) that the method may give widely divergent results even with crystalline 'B₁₂'. We found, in agreement with Shive, Ravel & Eakin (1948) and Wright, Skeggs & Huff (1948), that this *Lactobacillus* requires 'Tween 80' as well as tomato juice and anti-pernicious anaemia factor (APAF) for maximal growth on semi-synthetic media. The further observation by Wright *et al* (1948) that thymidine allows growth of *Lb lactis* Dorner ATCC 8000 on vitamin B₁₂-deficient media has been confirmed.

Shorb & Briggs (1948) have recently reported the method used for B₁₂ assay. We have been unable to obtain a response to APAF using their medium which appears to be deficient in growth factors required by our strain of *Lb lactis*. Shorb & Briggs (1948) state that variants of *Lb lactis* are readily formed, it is possible that the strains we obtained from the ATCC were all more exacting variants of this organism.

The cup plate assay (Bacharach & Cuthbertson, 1948) has been found adaptable to the determination of APAF activity and to the detection of other members of the B₁₂ group of microbiological growth

factors in purified liver extracts as reported in the next communication. The medium used is essentially that of Roberts & Snell (1946), with the addition of tomato juice, 'Tween 80' and 2% agar. For an assay the sterile medium is melted, held at 45°, and inoculated with a culture of *Lb lactis* Dorner ATCC 8000, 12.5 ml samples are then poured into Petri dishes. Holes are cut in the covered agar plates with a 10 mm cork borer. Three drops of test or standard solution are placed in each of the appropriate holes and the plates are incubated overnight. After 16–24 hr the colonies developing around the holes form sharply defined zones of exhibition. Under our conditions zone diameters are proportional to the logarithms of the APAF concentrations over the range 0.02–0.5 µg cryst APAF/ml. Microbiological assays and clinical trials of various liver preparations are being conducted, and it is hoped to report later on the comparisons.

Both anti-pernicious anaemia factors contribute to the microbiological activity. Unless the ratio of clinical to microbiological activity is the same for both of these substances this test alone will not exactly predict the clinical potency of liver extracts.

As a method of assay this has the advantage of speed and simplicity, but it is relatively insensitive and somewhat susceptible to interference by other members of the B₁₂ group, preservatives and antibiotics, though this is usually easy to detect.

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Chromatography of the Vitamin B₁₂ Group of Factors By W F J CUTHBERTSON and E LESTER SMITH (*Research Division, Glaxo Laboratories Ltd, Greenford, Middlesex*)

We have previously demonstrated the presence in liver extracts of two red substances clinically active against pernicious anaemia (Lester Smith, 1948, Lester Smith & Parker, 1948). One was obtained in crystalline form and is probably identical with 'vitamin B₁₂' (Rickes, Brink, Komuszy, Wood & Folkers, 1948). Shive, Ravel & Eakin (1948) have shown that liver extracts contain thymidine, and that this at relatively high concentrations simulates the microbiological activity of vitamin B₁₂.

A combination of partition chromatography on paper with microbiological assay on a solid medium permits the simultaneous demonstration of these three factors in liver extracts, together with a fourth microbiologically active component.

Water saturated *n*-butanol is the solvent used, with upward or downward development. Two techniques are available for observing the developed spots. A drop of adequately purified material, containing at least 10 µg of the factors, gives directly

visible red spots. The crystalline factor gives a single spot, but the mother liquors usually give a second fainter and slower-moving spot and occasionally a third, which travels fastest and appears to be a microbiologically inactive degradation product.

The other technique can be applied to fairly crude extracts and requires only 0.005–0.1 μ g of the factors; the developed strip is applied to the surface of nutrient agar seeded with *Lactobacillus lactis* Dörner (as used for the cup plate assay described in the preceding communication), being removed after 10 min, and the plate incubated overnight. The usual pattern is an ellipse of growth not far from the origin and another a few centimetres along, while much farther along, and beyond the position occupied by riboflavin (if present), are one or two zones of attenuated growth.

The first two zones are due to the two red factors, the third to a substance not yet characterized, and the fourth to thymidine. (We are grateful to Dr T. G. Brady of University College, Dublin, for a generous gift of thymidine.)

We have examined by this technique samples of the 'animal protein factor' of bacterial origin*. Two zones of strong growth and one or two of diffuse growth were always observed, and these were always in the same relative positions as the zones from liver extract.

In addition, preparations from three different bacterial strains, after purification by methods similar to those used for liver extracts, have revealed pink bands on silica partition chromatograms; on elution the microbiological activity was found to be concentrated in these pink bands.

We conclude that these bacteria elaborate the anti-pernicious anaemia factor and probably also the second active red factor, as well as thymidine. The term 'animal protein factor' may thus become redundant, especially since Stokstad, Page, Pierce, Franklin, Jukes, Heinle, Epstein & Welch (1948) have already demonstrated the activity of similar microbiological material against pernicious anaemia.

* One sample was kindly furnished by Dr T. H. Jukes of Lederle Laboratories Division, American Cyanamid Company.

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The Inhibition by Normal Horse Serum of (a) Trypsin and (b) a Proteolytic Enzyme of *Clostridium welchii* type B (λ -enzyme) By ETHEL BIDWELL (The Wellcome Research Laboratories, Beckenham, Kent)

The method of Oakley, Warrack & Warren (1949) for titrating sera for antibody to bacterial gelatinases has been found applicable to the measurement of trypsin and trypsin-inhibitor activities in terms of an arbitrary standard preparation of trypsin. The test is easy and gives reproducible results to within 10%. Phenolic preservatives do not interfere.

The trypsin-inhibitor values of sera were compared with their power to inhibit the action of a bacterial protease on 'azocoll' (hide powder, dyed with a red dye, Oakley, Warrack & van Heyningen, 1946). The bacterial enzyme used was the λ -antigen of *Clostridium welchii* type B which disintegrates azocoll, but not muscle or collagen 'paper', it is inhibited by normal horse serum (Oakley *et al.* 1949).

λ -Inhibitor and trypsin inhibitor values were determined in samples of (a) fresh normal horse sera, (b) sera which had been stored for several years, (c) sera which had been acidified to pH 4.0, incubated

at 37° for 0.5 hr and then neutralized, (d) fractions of normal horse sera obtained by salting out with ammonium sulphate.

The experiments showed that the overall trypsin-inhibitor and λ -inhibitor activities of normal horse serum are not identical, the λ inhibitor was more labile than the trypsin-inhibitor under all the conditions tried. The work of Schmitz (1938) and Grob (1946) and earlier workers strongly suggests that the 'anti-trypsin' of serum is complex. The possibility remains that one of the factors inhibitory to trypsin also inhibits λ enzyme. The trypsin-inhibitor was found to be more stable to ageing and acidification than the literature would lead one to expect. Three out of four sera, preserved with *o*-cresol and stored 6–10 years in a refrigerator, were still 40–70% as active in inhibiting trypsin as an average fresh serum. In experiments in which serum was fractionated with ammonium sulphate, not more than 50%

of the λ inhibitor could be accounted for, that which was recovered appeared almost entirely in the fraction precipitated between 15 and 30 % (w/v)

ammonium sulphate Trypsin-inhibitor was distributed between this fraction and the fraction soluble in 30 % (w/v) ammonium sulphate

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An Effect of L-glutamate on the Loss of Potassium Ions by Brain Slices Suspended in a Saline Medium By H A KREBS and L V EGGLESTON (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield*)

When slices of brain cortex were incubated aerobically in a saline medium containing about the same concentrations of inorganic ions as blood plasma the potassium ions of the tissue were found to diffuse into the medium. Addition of glucose to the medium reduced the loss of potassium by the tissue but did not prevent it. Many substances were added to the medium to test whether they can prevent the loss of potassium, and L-glutamate was the only substance found to have this effect. This is illustrated by the data given in the table

L Glutamate has been assumed for some time to

play a special role in the metabolism of brain and other tissues, because it is present in brain tissue in relatively large concentrations and is the only amino-acid which is readily oxidized in this tissue, but so far it has not been possible to define this special role. The present experiments indicate that glutamate takes part in the control of the ionic environment of nervous tissue. This result is of interest in connexion with recent experiments of Hodgkin & Huxley (1947) and of Keynes (1948), which suggest that potassium ions are ejected from nervous tissue on stimulation

Changes in the concentration of potassium ions in brain tissue and saline medium on incubation with different substrates

(About 100 mg (fresh weight) guinea pig brain cortex shaken for 60 min in physiological saline (Krebs & Henseleit, 1932) Gas 5% CO₂, 95% O₂ 40°)

Substrate added (final concentration)	None	Glucose (0.02M)	L Glutamate (0.005M), glucose (0.02M)	L Glutamate (0.005M)
Initial concentration of K in slices (mequiv/kg)	109	109	109	109
Final concentration of K in slices (mequiv/kg)	51	75	107	72
Initial concentration of K in medium (mequiv/l)	4.99	4.99	4.99	4.99
Final concentration of K in medium (mequiv/l)	6.34	6.01	5.01	5.61

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Determination of p-Aminosalicylic Acid in Body Fluids By J P NEWHOUSE and W KLYNE (*Postgraduate Medical School, London, W 12*)

Way, Smith, Howie, Weiss & Swanson (1948) have described a method for the determination of p-aminosalicylic acid in biological material using the reactions employed by Bratton & Marshall (1939) for the determination of sulphonamides. We were developing a similar method when the paper of Way *et al* appeared, and had already discovered conditions in which the diazotization and coupling could

be carried out at room temperature instead of at 0°, as used by them. We have subsequently introduced into our method one feature of Way's method, viz the addition of acetic acid at the coupling stage, which increases the intensity of the final colour.

Oxalated blood, plasma or cerebro-spinal fluid (0.2 ml) is measured into a test-tube containing 6.7 ml water. The contents of the tube are shaken

and allowed to stand 3 min. Trichloroacetic acid (0.6 ml of 25%, w/v) is added, after shaking, the mixture is allowed to stand 15 min and is then filtered through a small Whatman paper (no. 40 or 42) into a graduated centrifuge tube.

To 5.0 ml of filtrate, which must be clear, are added 1.5 ml conc. HCl and then 0.2 ml NaNO₂ solution (1%). The tube is shaken for 30–40 sec, and 1.0 ml of ammonium sulphamate reagent is added immediately. (The reagent consists of 2.0 g ammonium sulphamate in 50 ml glacial acetic acid and 50 ml water.) The solution is shaken for 10 sec, and 1.0 ml of naphthylethylenediamine dihydrochloride solution (0.2%) is added. The timing and shaking at these stages must be uniform. *p*-Aminosalicylic acid gives a purple colour which attains its maximum intensity after standing 15 min at room temperature and is stable for at least 3 hr. Its intensity is measured with a photoelectric photometer, using a yellow-green filter (e.g. Ilford no. 605).

The colour produced is compared with that obtained from a standard representing 10 mg *p*-aminosalicylic acid/100 ml blood, which is prepared

as follows. A stock solution is prepared containing 183.9 mg sodium *p*-aminosalicylate dihydrate/l, and 5.0 ml of this are diluted to 100 ml. To 2.0 ml of this solution are added 2.6 ml water and 0.4 ml of trichloroacetic acid, and the solution is treated as the 5.0 ml of blood filtrate.

The blank used to set the instrument to zero consists of 0.4 ml trichloroacetic acid and 4.6 ml water, treated as the 5.0 ml of blood filtrate. A blood blank is not usually necessary, since normal blood contains no significant quantities of interfering substances. The colour developed is directly proportional to the *p*-aminosalicylic acid concentration from 0 to 10 mg/100 ml blood, but this linear relationship does not hold strictly at higher concentrations.

When *p*-aminosalicylic acid (1–20 mg/100 ml) was added to normal blood or cerebro-spinal fluid the following recoveries were obtained: blood 77–94%, mean 85%; c.s.f. 90–100%, mean 94%. Duplicates agreed within about 0.3 mg/100 ml. Streptomycin did not interfere in the determination.

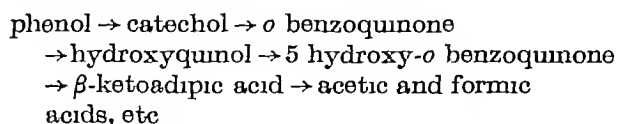
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The Determination of Bilirubin in Blood Plasma By E. J. KING and R. V. COXON

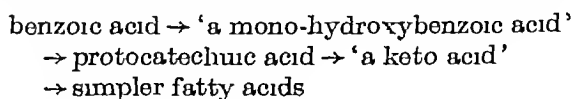
The Bacterial Oxidation of Aromatic Compounds By R. A. EVANS, W. H. PARR and W. C. EVANS (Biochemical Laboratories, Department of Animal Health, University College of Wales, Aberystwyth)

It has been shown (Evans & Happold, 1939; Evans, 1947; Kilby, 1948) that the bacterial oxidation of phenol by a vibrio (01), and other micro organisms isolated from soil and faeces, proceeds according to the following scheme:



The evidence for this metabolic pathway is based on chemical isolation of the intermediates in most cases, the gaseous exchange, and simultaneous adaptation.

Starting with benzoic acid as the sole carbon source, evidence has already been presented (Evans, 1947) for the following pathway in the bacterial oxidation of this compound:



We have now shown by chromatographic methods, followed by chemical isolation, that protocatechuic acid is decarboxylated giving catechol, which is then metabolized according to the phenol scheme, giving β -ketoadipic acid, etc.

From adaptation experiments, evidence is presented for the view that the first oxidation product of benzoic acid may be *p*-hydroxybenzoic acid, although this compound has not, as yet, been isolated from the bacterial culture. The experiments utilizing benzoic acid as substrate have been carried out with the vibrio (01), and *Pseudomonas fluorescens* strains A38 and A39, the latter cultures having been kindly supplied by Dr Stanier, Department of Bacteriology, University of California, U.S.A.

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The Effect of the Inclusion of Bracken (*Pteris aquilina*) in the Diet of Rats By E T R EVANS and W C EVANS (*Department of Animal Health, University College of Wales, Aberystwyth*)

Rats fed on a standard basal diet consisting of

B D H Light White Sol Casein	20 parts
Sucrose	60 "
Arachis Oil	15 "
B D H 'Dunn' Salt Mixture	5 "
Dried Yeast	10 "

(supplemented with radiostoleum, and α -tocopherol), together with the addition of heat dried, ground bracken leaves (collected in July, when at maximum growth), in the ratio of 60 % basal diet to 40 % bracken powder, eventually developed symptoms of B₁ deficiency. Unless treated, they lost weight and died within 1 month, the animals respond specifically to thiamine therapy. This con-

firms the short note (with no details of diet) of Weswig, Freed & Haag (1946) on the anti-thiamine activity of certain plant materials.

Some evidence incriminating the 'tannin' fraction of the bracken was discussed, as playing some role antagonistic to B₁. Preliminary growth experiments utilizing *Phycomyces blakesleeanus* shows that tannic acid exerts an inhibitory action on the growth of this fungus, at fairly low concentration, in a medium which is otherwise completely adequate for growth.

Bracken poisoning of farm animals is well known in certain parts of this country, and the implications of these experiments (including B₁ therapy) were discussed.

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The Intestinal Conversion of Carotene to Vitamin A By S Y THOMPSON, R BRAUDE, A T COWIE, J GANGULY (Government of India Scholar) and S K KON (National Institute for Research in Dairying, University of Reading)

It is now established that carotene is converted to vitamin A in the small intestine of the rat (Glover, Goodwin & Morton, 1947, Thompson, Ganguly & Kon, 1947, Mattson, Mehl & Deuel, 1947) and of the pig (Thompson *et al* 1947). We have only recently learnt that Wagner (1939) holds a similar view about the whale.

In our experience the presence of fat is not essential for the absorption of vitamin A acetate by the rat. The acetate was added as a solution in *n* hexane to a diet deficient in vitamin A previously exhaustively extracted with diethyl ether, and the hexane was allowed to evaporate. β -Carotene simi-

larly added to the fat-free diet as a solution in diethyl ether was converted to vitamin A only to a very small extent. When, however, the carotene was dispersed in water by means of acetone in the way described by With (1939) and the colloidal solution was given, mixed with the fat-free diet, to vitamin A deficient rats, the intestinal conversion to vitamin A and the absorption of the vitamin A were nearly as efficient as with oily solutions (see table). Colloidal solutions of vitamin A prepared by the same method were also well absorbed.

Experiments with pigs deprived of vitamin A, with London cannulas in the portal vein or in its

The appearance of vitamin A in the small intestine, blood and liver of vitamin A-deficient rats after a meal of 3.5 mg of β carotene in colloidal or oily solution

Mean values in i.u./rat

Time after dosing (min)	Small intestine		Blood				Liver	
			Colloidal carotene		Carotene in arachis oil			
	Colloidal carotene	Carotene in arachis oil	Alcohol	Ester	Alcohol	Ester	Colloidal carotene	Carotene in arachis oil
0		3 (5)*	0.7	0.4	0.7	0.4		
15	14 (12)*	14 (6)*	0.8	0.3	0.7	0.4		
30	28 (9)*	25 (6)*	0.5	0.3	0.6	0.3	2	2
60	51 (3)*	66 (2)*	1.4	2.0	1.6	2.0	3	2
120	54 (3)*	119 (2)*	5.4	1.8	5.2	7.0	14	7
							40	67

* The figures in brackets indicate the number of rats on which values for intestine, blood and liver are based

mesenteric branch, showed, in agreement with the findings of Goodwin, Dewar & Gregory (1946) for the sheep, that after a meal of carotene in oil little or no carotene could be detected in the systemic or portal blood. Vitamin A ester appeared in increased quantities in the blood within 2 hr of the carotene meal, an increase in vitamin alcohol followed about 2 hr later. Vitamin A, exclusively in the ester form, appeared within 75 min in the lymph from the duct draining the mesenteric lymphatics and

in the wall and contents of the small intestine. Under ultraviolet light the mesenteric lymphatics showed the characteristic yellow fluorescence of vitamin A.

We are indebted to Dr O. Isler of Hoffmann La Roche, Basle, for the gift of synthetic vitamin A acetate, to Dr M. Van Eekelen, Utrecht, for advice on the preparation of colloidal solutions of carotene, and to Dr C. E. Dent, University College Hospital Medical School, for the loan of a cannula and advice on its use.

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The Pyruvate Oxidase System in Pigeon Brain and the Tricarboxylic Acid Cycle. By R. V. COXON*, C. LIÉBECQ† and R. A. PETERS (Department of Biochemistry, University of Oxford)

Using dialyzed homogenates from pigeon brain prepared by grinding in the ice-cold mortar (previously called 'dispersions' by Banga, Ochoa & Peters, 1939), it has been found that citrate and an α -keto acid presumed to be α -keto glutarate accumulate during the oxidation of pyruvate in the presence of fumarate. In absence of fumarate there is little accumulation of these two substances, though a greater formation of acetate occurs. Hence though citrate does not act catalytically, the fact that it appears during pyruvate oxidation when fumarate is present is support for the participation of the Krebs tricarboxylic acid cycle and for the idea that the pyruvate oxidase system in brain tissue, as understood in this laboratory, includes a major part of this 'cycle'. We assume that the citrate is formed from *cis*-aconitate according to accepted theory. In our preparations there appears to be a block in the

enzymes of the 'cycle' at the tricarboxylic stage, this accounts for the failure of citrate to increase oxygen uptake as does fumarate (Banga *et al* 1939) and for the accumulation of citrate in the present experiments. On the basis of such a block the reasons for the appearance of α -ketoglutarate are not yet clear, especially as it is also known that this dicarboxylic acid can be oxidized by our preparations.

Since the Neuberg-Case methods of pyruvate estimation do not distinguish between pyruvate and α -ketoglutarate 2,4-dinitrophenyl hydrazones, our estimations of these substances have been made by the method of Friedemann & Haugen (1943), using the Beckman spectrophotometer, and have been checked by conversion of α -ketoglutarate to succinate (Krebs, 1938) and independent determination of pyruvate by the Straub method (1936).

* Betty Brookes Fellow

† British Council Scholar

We are grateful to R. W. Wakelin for technical assistance.

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The Nature of the Prothrombin Deficiency in Dicoumarin Plasma By C A MAWSON (*Pathological Laboratory, Royal Berkshire Hospital, Reading*)

When prothrombin is estimated in the plasma of patients receiving dicoumarin, results obtained by the one stage method vary with the thromboplastin used. Venom-lecithin gives higher and ox-lung extract lower results than those obtained with dried rabbit brain. The two stage method, which is generally regarded as more reliable, gives results which agree fairly well with those found by the venom-lecithin one stage method, but are substantially higher than those obtained with brain. When normal plasma is mixed with dicoumarin plasma the brain results no longer differ greatly from those obtained by the venom lecithin and two stage methods. This suggests that normal plasma and venom lecithin each contain something which is necessary for the estimation of prothrombin by the one stage method, but which is deficient in brain and in dicoumarin plasma.

The function of this substance (or substances) seems connected with the rate of conversion of prothrombin to thrombin. When a discrepancy exists between the brain and venom-lecithin results in dicoumarin plasma, the prothrombin conversion time is greater than normal, but when the drug is stopped and the conversion time returns to normal the discrepancy disappears. Sometimes the prolongation of the conversion time is delayed and the discrepancy does not occur until the conversion time increases. These facts cannot be explained by deficiency in Factor 5 (Owren, 1947) because dicoumarin plasma

contains a normal amount of this substance. It is no less efficient than normal plasma in restoring the activity of normal plasma in which Factor 5 has been decreased by ageing. Furthermore, plasma deficient in Factor 5 gives lower results with venom-lecithin than with brain, while the reverse is true for dicoumarin plasma.

A patient with non-tropical sprue, who had a very low plasma prothrombin and multiple haemorrhages, was treated with a vitamin K analogue. The prothrombin rose overnight to 100% as measured by the brain one stage method and continued at or above this level until death a week later, but although the haemorrhages decreased they did not cease. The venom-lecithin method never gave a result higher than 61% (mean 47%), while the two-stage method gave a maximum of 62% (mean 53%). The situation appeared to be exactly opposite to that found in dicoumarin plasma, for the conversion time of 1/50 plasma before treatment was 10 min, which fell within 12 hr after giving vitamin K to 2 min. However, mixtures of the treated sprue plasma with dicoumarin plasma consistently failed to show that it was more efficient than normal plasma in abolishing the discrepancy between the results obtained by the use of venom-lecithin and brain in the one stage method.

This work suggests the existence of accelerators of the conversion of prothrombin to thrombin, other than Factor 5.

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DEMONSTRATIONS

Ancillary Apparatus for Chromatography (a) Mechanical Fraction Cutter, (b) Conductivity Recording Apparatus, (c) Method of Packing Kieselguhr Partition Columns with Aid of Perforated Plate on Long Wire Handle By S. S. RANDALL and A. J. P. MARTIN

A Device for the Rapid Calculation of the Molecular Rotations of Steroids By W. KLYNE (*Postgraduate Medical School, London, W 12*)

Barton & Klyne (1948) have published tables of the molecular rotations $[M]_D$ of certain fundamental steroid structures and the molecular rotation contributions (Δ values) of substituents, which are characteristic of the nature and position of the substituents. These Δ values are generally speaking in-

dependent of one another, unless the substituents are close together and exert vicinal action on one another (cf. Barton & Cox, 1948).

The $[M]_D$ values of compounds where vicinal action is absent can be calculated by adding together the $[M]_D$ value of the fundamental structure

and the Δ values of the substituents. The calculating device consists of a series of concentric graduated discs on which are marked the fundamental $[M]_D$ values and the Δ values (ΔE , ΔO , ΔA and ΔK values for olefinic double bonds and for hydroxyl, acetoxy and keto groups respectively). These values can be added together by turning the discs about their common centre in the manner of a circular slide rule.

The extent of vicinal action in compounds where

substituents are close together may be estimated by measuring the difference between the $[M]_D$ value calculated from the standard values in the tables, and the experimentally observed $[M]_D$ value.

The application of the molecular rotation difference method and the use of the calculating device in helping to elucidate the structures of naturally occurring steroids was illustrated by examples. The use of the calculating device in the study of vicinal action was also demonstrated.

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The Determination of Plasma Iron with the Beckman Spectrophotometer By I. D. P. WOOTTON and J. C. S. PATERSON

A Capillary Method for Determining the CO₂ Combining Power of Plasma By I. D. P. WOOTTON and E. J. KING (Postgraduate Medical School, London, W. 12)

This rapid method, which requires only a small amount of plasma, has been found useful in estimations of the CO₂ combining power of animal and human blood, and as a field method during the cholera epidemic in Egypt last year. The plasma, equilibrated with alveolar air, is mixed with an equal quantity of a standard sulphuric acid and by trial the concentration of acid required to reduce the pH of the mixture to 5.5 is found. This procedure was developed from that described by Wright & Colebrook (1921).

Apparatus and reagents (1) Capillator set for chlorophenol red (British Drug Houses) (2)

Standard sulphuric acids of various concentrations. These are prepared by dilution of standard N/10 acid, and are preserved in waxed bottles. For most purposes the following series of strengths is sufficient.

N/20, N/25, N/30, , N/55, N/60

(3) Chlorophenol red solution, 0.04%, in aqueous solution. (4) Light blue optical filter, e.g. Ilford 303. (5) Plasma or serum, equilibrated with alveolar CO₂.

Procedure A small Pasteur-type pipette is prepared by fitting a bulb to a capillary tube. It is

Table 1. Some of the parallel gasometric and titrimetric estimations used to find the empirical factor

No	(A) Observed CO ₂ combining power (Van Slyke)	(B) Normality of acid to reduce pH to 5.5	(C) Factor to reduce normality to CO ₂ combining power ($C = A/B$)	(D) Calculated CO ₂ combining power (using average factor) ($D = \bar{C} \times B$)
1	40	N/30	1200	38
2	30	N/40	1200	29
3	35	N/35	1225	33
4	48	N/25	1200	46
5	63	N/20	1260	58
6	50	N/25	1250	46
7	10	N/100	1000	12
8	10	N/100	1000	12
9	30	N/40	1200	29
10	22	N/50	1100	23

Average factor from all determinations = 1165 (\bar{C})

graduated by marking the tube at about 1 and 2 in from the open end, i.e. to contain 1 or 2 volumes. Two volumes of chlorophenol red solution are withdrawn and discharged into a watch-glass, or on to a waxed slide. Using the same pipette, one volume of one of the standard acids, followed by one volume of serum or plasma, is withdrawn, added to the indicator and well mixed.

The pH of the mixture is ascertained by drawing it up into the pipette and comparing it with the standard tubes on the card. The comparison is facilitated by viewing the tubes by transmitted light through the blue filter.

After washing the pipette in distilled water the procedure is repeated with another acid until the concentration of acid is found which will give a final pH of 5.5.

Calculation CO_2 combining power in vol/100 ml $= 1165 \times \text{normality of acid required to reduce pH to 5.5}$, i.e. if acid was $N/35$, then CO_2 combining power $= 1165 \times 1/35 = 33$. The figure 1165 is an empirical factor (see Table 1) found by comparing titrations with gasometric results. A series of parallel determinations has shown that the standard deviation of the titration results from the gasometric results is ± 4 vol/100 ml.

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The Determination of Bilirubin in Blood Plasma By E. J. KING, R. V. COXON, L. ROSS and M. LEVINE

The Determination of Blood Constituents with the M. R. C. Grey Wedge Photometer By E. J. KING and P. ROSENBAUM

Attempts to Prevent Experimental Silicosis with Aluminium By E. J. KING, B. M. WRIGHT, S. C. RAY and C. V. HARRISON

Case Records of Patients Treated with Dicoumarin By C. A. MAWSON

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*

- The influence of the pituitary on phosphorus metabolism of brain By M REISS, F E BADRICK and JEAN H HALKERSTON
- Studies on cholinesterase 6 The selective inhibition of true cholinesterase *in vivo* By ROSEMARY D HAWKINS and B MENDEL
- The absorption of vitamin A in ruminants and rats By E EDEN and K C SELLERS
- The decarboxylation of o hydroxyphenylalanine By H BLASCHKO
- Reducing-group production from starch by the action of α - and β amylases of barley malt Activity of α - and β amylases By I A PREECE and M SHADAKSHARASWAMY
- Changes in the extracellular and intracellular fluid phases of muscle during starvation and dehydration in adult rats By S E DICKER
- Colorimetric determination of potassium by Folin-Ciocalteu phenol reagent By M A M ABUL FADL
- The nicotinamide saving action of tryptophan and the biosynthesis of nicotinamide by the intestinal flora of the rat By P ELLINGER and M M ABDEL KADER
- The measurement of glucuronide synthesis by tissue preparations By G A LEVVA and I D E STOREY
- The frequency distribution of the zinc concentrations in the dental tissues of the normal population By D B CRUICKSHANK
- A biochemical study of *Pseudomonas prunicola* Wormald 1 Pectin esterase By G BARBARA MILLS
- The measurement of the cytochrome oxidase activity of enzyme preparations By E C SLATER
- Studies on the absorption of proteins the amino acid pattern in the portal blood By C E DENT and J A SCHILLING *Addendum* Conjugated amino acids in portal plasma of dogs after protein feeding By H N CHRISTENSEN
- The metabolism of sulphonamides 6 The fate of some N^4 n acyl derivatives of ambamide (Marfanil) and the sulphone, V335, in the rabbit By R L HARTLES and R T WILLIAMS
- The fluorimetric estimation of riboflavin in foodstuffs and other biological material By E KODICEK and Y L WANG
- The fluorimetric estimation of nicotinamide in biological materials By D K CHAUDHURI and E KODICEK
- The tryptophanase tryptophan reaction 9 The nature, characteristics and partial purification of the tryptophanase complex By E A DAVES and FRANK C HAPFOLD
- Metabolism of polycyclic compounds 5 Formation of 1 2 dihydroxy-1 2 dihydronaphthalenes By JOAN BOOTH and E BOYLAND
- The excretion of synthetic oestrogens as ethereal sulphates and monoglucuronides in the rabbit and in man By S A. SIMPSON and A E WILDER SMITH
- The formation of hydrogen carriers by haematin-catalyzed peroxidations 2 Some reactions of adrenaline and adrenochrome By J E FALK
- The substrate specificity of the tyrosine decarboxylase of *Streptococcus faecalis* By G H SLOANE STANLEY
- The effects of applied pressure on secretion by isolated amphibian gastric mucosa By R E DAVIES and C TERNER

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 273rd Meeting of the Biochemical Society was held at the Westminster Hospital Medical School, 17 Horseferry Road, London, S W 1, on Saturday, 12 February 1949, at 11 a m., and took the form of a Symposium on 'Biochemical Aspects of Genetics'

COMMUNICATIONS

Introduction By J B-S HALDANE (University College, London, W C 1)

Genetical research has revealed the presence in cells of at least two types of self-reproducing units, nuclear units or genes, and extranuclear units. Both are capable of self-reproduction for an indefinite period, though liable to be lost, duplicated, or changed into a new self-reproducing form. In bacteria the distinction becomes uncertain, since a unit is usually only assigned to the nucleus because it is inherited according to Mendel's laws or some modification of them in sexual reproduction.

Three questions have to be answered concerning each such unit. What is it, what does it do, and how is it reproduced, with or without alteration? Roughly speaking, we may say that each gene is responsible, not for a unit character, such as a form or colour, but for a unit biochemical process. It is possible that antigens are immediate products of gene action, but in general when a gene is responsible for a step in a synthesis it probably acts by catalyzing the synthesis of an enzyme. Besides controlling catalysis, genes may control membrane permeability, and doubtless many other biochemical processes.

The chromosomes are generally believed to consist mainly of deoxyribonucleoprotein. The gene-like bacterial transforming substances discovered by Avery, Macleod & MacCarty (1944), Boivin, Vendrely & Lehault (1945) and their colleagues occur in highly purified deoxyribonucleic acids. If, as seems likely, there is no sharp line between the extranuclear units and molecular viruses, these may consist of either type of nucleoprotein. It is an attractive speculation, though at present no more, that the fundamental pattern of a gene can be expressed either in nucleic acid or protein, and copied from one to the other.

Any interference with the normal self-reproductive process is called mutation. In *Drosophila* spontaneous mutation has a fairly high temperature coefficient, and is therefore a biochemical process. Mutation can be produced by a large variety of

biochemical agents ranging from formaldehyde through mustards and carcinogens to antibodies. Since X-rays can produce mutation not only by direct action but by action on the culture medium (Stone, Haas, Clark & Wyss, 1948), and in one case at least (Thoday & Read, 1948) are three times as mutagenic in the presence of oxygen as in its absence, it seems likely that mutation is always a biochemical rather than a biophysical process. In some cases the genes are changed directly. In others the interference may be with their reproductive process.

Some of the extranuclear units reproduce in a variety of conditions. Others are formed in the presence of certain genes if the environment is favourable, but can then be reproduced indefinitely, even in the absence of the genes concerned. This is the case with adaptive enzymes in yeast, responsible for the fermentation of galactose and melibiose (Spiegelmann, Lindegren & Lindegren, 1945), which persist so long, but only so long, as these sugars are supplied. It is not certain whether the self-reproducing units are the enzymes or systems responsible for their production. The former is the simpler hypothesis. If there are catalysts which reproduce when they catalyze, as there are others which are used up in this process, we may expect that abnormal physiological activity or inactivity of genes may induce them to mutate in a manner which would be on the whole adaptive.

Geneticists should be able to offer to biochemists races of the same species differing only in respect of one particular biochemical process. It is for the biochemists to discover what that process is. Since geneticists commonly detect gene differences on the basis of morphological differences, this means, among other things, that the biochemistry of some critical steps in morphogenesis can be investigated. On the other hand geneticists are likely to reach far greater precision in their work by using biochemical criteria wherever possible.

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Genetic Control of Biochemical Synthesis as Exemplified by Plant Genetics—Flower Colours.

By W J C LAWRENCE (*John Innes Horticultural Institution, London, S W 19*)

In 1909, Onslow, working with *Antirrhinum majus*, made the first chemico-genetical investigation on flower colour and put forward the suggestion that anthocyanins were derived (sequentially) from anthoxanthins. With this exception, the work of biochemists and geneticists was pursued separately until about 1930. Each group had by then accumulated sufficient knowledge and adequate techniques to enable joint studies to yield rapid and illuminating results. Scott Moncrieff, at Haldane's suggestion, and later Price, working with the geneticists at the John Innes Institution, showed that anthocyanin production, amount, state of oxidation, degree of methylation and glycosidal type were each determined by simple gene relationships. Flavone and chalcone production and amount were found to be

similarly governed. A further advance was the discovery in *Dahlia* that there was competition in the production of anthocyanin, flavone and chalcone, according to gene dosage, implying parallel production from a common precursor. The theory advanced for pigment production in *Dahlia* was found to support the important suggestion by Robinson, that different anthocyanins, etc., were derived from the same intermediate, built up from two hexose and one triose unit by a series of aldol condensations and dehydrations. On this hypothesis, cyanidin is synthetically the simplest anthocyanin. It is also, in fact, the most commonly occurring anthocyanin in flowers and leaves, especially in the least highly evolved flowering plants.

Garrod's Conception of Inborn Error and its Development By L S PENROSE (*The Galton Laboratory, University College, London, W C 1*)

Garrod emphasized that biochemical differences could be found between members of the same species as well as between individuals of different species. He examined alcaptonuria and other rare conditions in which specifically abnormal metabolism could be detected both from the clinical and genetical points of view. He was able to demonstrate that rare Mendelian recessive genes would give rise to the familial appearances, which are found in most of these conditions. The analysis enabled him to explain for the first time the special significance of inbreeding in man. This new biological approach laid the foundation for an understanding of metabolic abnormalities in man on the supposition that single gene differences between individuals could determine not only primary abnormalities like albinism and alcaptonuria but also secondary clinical effects such as are shown in diabetes.

It is convenient to separate those conditions

determined by a gene in homozygous recessive form from those in which the causal gene is heterozygous (incompletely recessive). The homozygous conditions, which include albinism, pentosuria, fructosuria, phenylketonuria and (in many cases) alcaptonuria, cystinuria, lipoidoses, methaemoglobinuria, are constant in their manifestation throughout life. The heterozygous conditions are clinically more variable and may first develop in adult life as in acute porphyria and some types of cystinuria. It would be of great interest to know the nature of the homozygous forms of these incompletely recessive conditions. Conversely, the carriers of recessive abnormalities may sometimes show mild peculiarities.

The scope of Garrod's idea is very wide, and it may be said that it is now a fundamental principle in human genetics to express inherited individual differences in terms of biochemistry.

The Interpretation of Biochemical Detail Revealed by Inborn Errors By C RIMINGTON
(University College Hospital Medical School, London, W C 1)

Inborn metabolic error, according to modern usage, may be described as a deviation from the metabolism normal to the species, manifesting itself either sooner or later in the life of the individual and referable to the genetic composition of the latter. Garrod clearly interpreted the metabolic errors he investigated as being due to the congenital absence of some particular enzyme required to complete a step of normal metabolic change. This gave rise to an accumulation of the intermediate product.

It is felt that other possibilities have to be borne in mind, as, for example, that the error may be such as to give rise to a product of quite foreign structure, a metabolic perversion, or to a failure in excretion or absorption. In the latter case it is not necessary to postulate any defect in intermediate metabolism *per se*.

The three known inborn errors affecting the aromatic amino-acids, phenylketonuria, tyrosinosis and alcaptonuria, are probably examples of metabolic arrest in Garrod's sense, although there is some suggestion that a renal function may be partly, at least, responsible for alcaptonuria.

The excretion of uric acid by the Dalmatian dog is an excellent example of metabolic anomaly which has been proved to be of entirely, or almost entirely, renal origin, the Dalmatian being unable to reabsorb uric acid from its glomerular filtrate (Friedman & Byers, 1948). Although its tissues contain as much uric acid as other dogs, the substrate and enzyme are apparently not in contact long enough for production of normal quantities of allantoin. It is pointed out that the inability to retain or absorb uric acid

may extend to cells of the body other than the tubule cells, and this consideration becomes of importance when trying to interpret the data of other metabolic anomalies.

In cystinuria, the urine contains besides cystine a pattern, variable from case to case, of other amino-acids, among which leucine and lysine are prominent.

It has been suggested by Dent (1948) that the error in this condition is a renal one, the absorption of a group of substances by the tubule being affected. Such an hypothesis receives support from the effect of administered leucine in raising the output of cystine, no doubt by competing with cystine for reabsorption by a particular group of tubule cells. Certain cases have been recorded—usually infants or young children—in which a condition of renal rickets was present, and after death extensive deposits of cystine were found in the internal organs. Cystinuria has rarely, if ever, been proved in these cases which may be examples of a different disease entity, 'cystine storage disease' (Russell & Barrie, 1936). Abderhalden's (1903) case was possibly of this type. Alternatively, one might suppose, as did Garrod, that an initial cystinuria can so damage the glomeruli that renal function fails and the unexcreted products become deposited in the tissues of the body.

It is felt that the few examples chosen indicate that the interpretation of biochemical detail presented by any genetically controlled anomaly demands a survey on a broader physiological basis than that exemplified by Garrod's conception of inborn metabolic error.

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Chemical Induction of Mutation By J G CARR (Chester Beatty Research Institute, London, S W 3)

Differences in the rate of spontaneous mutation were long known to arise in different physiological conditions, indicating that chemical influences could be active. But only in the last few years have chemicals been discovered which will act directly with the chromosome materials to yield new genetic forms. The four main types of compounds are

1 'Mustard' compounds, discovered by Auer-

bach and Robson. These are active in most species tried, and have been studied in most detail.

2 Carcinogenic hydrocarbons, first reported by Strong as active in mice. These are probably inactive when fed.

3 Carbonyl compounds, studied by Rapoport in *Drosophila*, these may be active only when mixed with the food, and an altered food constituent may be the active material.

4 Phenolic compounds, studied by various workers, and another group of miscellaneous compounds

So far, all compounds are 'radiomimetic', i.e. they react apparently indiscriminately with all parts of the chromosome, and differ from the action of radiations quantitatively rather than 'qualita-

tively. The mode of reaction with the genetic material is unknown, and no compound reacting with only one gene has yet been obtained.

These chemicals are already of importance, not only in genetics, but also, for example, as a substitute for radiation in some kinds of malignant disease.

Gene Action and Mutation By D. G. CATCHESIDE (*Botany School, Downing Street, University of Cambridge*)

The biochemistry of the gene, particularly as regards the nature of mutation, can hardly at present be studied more closely than at third hand. It is generally accepted that specific genes mediate the production of specific enzymes which in turn catalyze specific metabolic processes. Normally, a mutation is detectable only by a change in the specific metabolic process catalyzed, and the type of gene change can be specified at best only in terms of probable differences in the enzymes produced. In cases where a series of multiple alleles of a specific gene is available, some are found to determine the same specific metabolic process, but under more restricted environmental conditions than is characteristic of the wild type and to be inactive under other conditions. Such mutations are to be understood as gene changes which leave the gene still able to mediate the production of an enzyme with the particular specificity

but with more restricted stability, for example, as regards temperature or pH. More extreme mutations show the loss of activity under all conditions within which the organism is viable. Some of these are able to revert, or back mutate, to wild type. In these, an almost intact gene capable of reproduction must persist, and we may postulate for them either unchanged specificity coupled with total loss of enzyme stability or a change or loss of specificity with or without stability changes. The former are probably much the commonest. The latter have not been observed, perhaps mainly because of technical difficulties. Others of the extreme mutations appear to be total losses of the enzyme, since back mutations never occur, and in some cases there are other indications of genetic deficiency. Most artificial mutagenic agents appear to induce the more extreme types of alteration.

New Fields in the Biochemical Genetics of Micro-organisms By G. PONTECORVO (*Department of Genetics, the University, Glasgow, W. 2*)

Recent techniques bring within the scope of genetics a range of micro-organisms including yeasts, moulds with and without a sexual stage, bacteria, bacteriophages and Protozoa. These techniques, and in particular that of Beadle and Tatum for the isolation of strains with hereditary blocks in the synthesis of essential metabolites, make possible the investigation of the genetical control of metabolic processes in micro-organisms, an investigation which was confined to higher organisms up to ten years ago. Since micro-organisms lend themselves to more extensive, and often more detailed biochemical analysis, a far more precise knowledge of the correlations between genetical architecture and biochemical activities is now on the way. The former consists of spatial patterns which the geneticist can analyze almost down to macro-molecular detail, knowing, however, next to nothing about their chemical basis, the latter consist of series of chemical reactions, which the biochemist can analyze in detail as to their steps and

their time sequence, knowing, however, next to nothing about their spatial organization. The gap between the two has somewhat narrowed following the discovery that, in certain cases at least, each of the units of genetical pattern—each gene—seems to control specifically, via an enzyme, a simple step in a chain of biochemical reactions.

A crucial problem, open to immediate experimental attack, is whether the organization of the genes in space and the time sequences of biochemical steps which they control are in any way connected, as are operations along assembly lines. There is some suggestion of this in the genetics of higher organisms, and two examples are known in *Neurospora*, but they may be no more than fortuitous. A search for close linkage between the genes controlling consecutive steps in one and the same chain of biochemical reactions is certainly worth making, in the writer's view the two most promising types of reactions are those of millimicromolar order (vitamin-like sub-

stances), and those for the synthesis of specific polypeptides, polysaccharides and polynucleotides, which also may be of millimicromolar order. For both, exploratory work is in progress in our laboratory.

In addition to these finest details of spatial pattern in biochemical processes, the investigation of which is now possible but may well prove to be based on wrong assumptions, the genetics of micro-organisms provides means for studying cruder types of bio-

chemical organization, e.g. localization of reactions in certain parts of the cell, concentration gradients, permeability of nuclear and cell membranes, etc. Essentially, the technique consists in observing the course of two reactions when the allelomorphs of two genes, controlling these reactions are arranged in the *trans*-(*Ab/aB*) as compared with the *cis*-(*AB/ab*) configuration between two cells, two nuclei within a cell or two chromosomes within a nucleus. Examples of this technique were given.

Adaptation, Mutation and Segregation in Relation to the Synthesis of Enzymes by Bacteria

By JACQUES MONOD (*Pasteur Institute, Paris, France*)

The assumption that simple relations exist between genes and enzymes is explicit or implicit in all the work on biochemical genetics.

In trying to specify and define this hypothesis one is led to consider the appealing idea that these relations may be the simplest possible, namely, one to one. This was proposed by Beadle and substantiated by the work of his group on genetic control of the biosynthesis of essential metabolites.

The great measure of success achieved by investigations based on this hypothesis suggests a further, more stringent one: why not assume identity between the specific gene and the specific enzyme assumed to control a given biosynthetic reaction? Whether right or wrong, this hypothesis, proposed by McIlwain, is certainly useful, since it leads to the interesting experiments reported by Dr Pontecorvo.

It should be noted, however, that these developments are almost entirely based on consideration of biosynthetic reactions studied with living cells, and assumed to be brought about by specific enzymes. It is evident that in most cases the enzymes are defined exclusively by the specificity of the genetic differences, so that one might be led to considering the properties of circular reasoning.

At this point it should be remembered that *specific*

factors other than genetic differences are known to control the synthesis of certain enzymes by micro-organisms. Such is the case for numerous *adaptive* enzymes, formed by the cells exclusively in the presence of a specific substance, the substrate of the enzyme. For instance, in *E. coli* ML, lactose is split by a typical *lactase*, and maltose is attacked by another specific enzyme, *amylomaltase*. Both enzymes have been extracted from the cells, and partially purified. Both have been shown to be present only within cells cultivated in the presence of each substrate. On the other hand, *genetic* control of these two enzymes can also be demonstrated by a study of spontaneous and induced mutations. The mutations show independence and specificity. Furthermore, using Lederberg's strain, recombination of the maltose and lactose characters can be demonstrated, and it would seem that the phenomenon is not affected by the 'state of adaptation' of the cells.

These observations are entirely compatible with the 'simple relation' hypothesis. They are hardly compatible, however, with the 'identity' hypothesis. In any case they tend to show that both genetic and external specific factors interact in the synthesis of specific enzymes.

The 274th Meeting of the Biochemical Society was held at St Thomas's Hospital Medical School, London, S E 1, on Friday, 18 February 1949, at 2 p m, when the following papers were read

COMMUNICATIONS

Non-nucleic Acid Protein-bound Phosphorus By J N DAVIDSON, MARY GARDNER, W C HUTCHISON, W M MCINDOE, W H A RAYMOND and J F SHAW (*Biochemistry Departments of St Thomas's Hospital Medical School, London, S.E 1 and The University, Glasgow, W 2*)

When a tissue is extracted with acid and then with lipid solvents so as to remove acid-soluble P and lipid P, it is generally assumed that the protein-bound phosphorus which remains is derived from ribonucleic acid and deoxyribonucleic acid. This protein bound fraction also includes phosphoprotein P which is frequently taken to be negligible in animal tissues (cf Schmidt & Thannhauser, 1945). If this assumption is correct the specific activity of the protein bound P fraction obtained from the tissues of rats and rabbits receiving P^{32} should lie between the values for the specific activities of the P in the isolated nucleic acids. We have found,

however, that the activity of the protein-bound P is very much greater than that in the P of either of the nucleic acids. An active fraction is liberated as inorganic P when the extracted tissue residue is incubated with alkali and is therefore presumably present in the tissue as phosphoprotein P. Although the amount of P in this fraction is minute, its activity is very high. This active fraction has been found in liver, spleen, thymus and intestinal mucosa and appears to be present in both cytoplasmic and nuclear material. Its occurrence has been demonstrated in isolated liver-cell nuclei.

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Further Studies on the Porphyrins of Acute Porphyria By R R McSWINEY, R E H NICHOLAS and F T G PRUNTY (*Departments of Chemical Pathology, St Thomas's Hospital Medical School, London, S E 1 and University College Hospital Medical School, London, W C 1*)

Additional confirmation has been obtained of the fact that the uroporphyrin of acute porphyria is mainly a type I isomer.

Studies of a further case have been made showing in an attack the excretion of up to 2.8 and 4.3 mg/day of coproporphyrin in urine and faeces respectively, and of 35 and 2 mg/day of uroporphyrin in urine and faeces respectively. Chromatographic separation of the porphyrins on alumina have shown the urine and faeces to contain similar porphyrins.

Paper chromatography has shown that the porphyrin, m p 227°, has probably five carboxyl groups

and indicates the presence of minute amounts of another porphyrin, not isolated, with seven carboxyl groups.

	Urine	Faeces
Coproporphyrin	I	I, small amounts of III present
Undentified porphyrin	m p 227-232°	m p 222-227°
Uroporphyrin	m p 271-274°	m p 272-275°

The uroporphyrin appeared to be a type B porphyrin of Watson's classification, which on decarboxylation yielded mainly coproporphyrin I with evidence of small amounts of III.

Studies on the Effect of the Substitution of Deuterium in the Methylene Groups of Succinic Acid on the Action of Succinic Dehydrogenase By M B THORN* (introduced by F G YOUNG) (*Department of Biochemistry, University College, London*)

The widespread use of deuterium as a tracer isotope in biological experiments appears to have been based largely on observations showing that concentrations

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of D_2O of up to 10% had no influence on a variety of biological and biochemical processes (e.g. Dungen, 1936). Theoretical calculations by Eyring & Sherman (1933), however, indicated that in chemical reactions at ordinary temperatures deuterium would react at

least 50 % more slowly than hydrogen. These calculations have been supported by a great deal of experimental work on relative rates of proton and deuterium transfer (e.g. Wynne-Jones, 1934, Wilson, 1936, Bonhoeffer, 1938), and it is therefore of interest and importance to inquire more deeply into the justification for the use of deuterium as a tracer isotope. The experiments reported here were undertaken in an attempt to determine the effect of the substitution of deuterium for hydrogen in a substrate on the action of an enzyme.

The preparation of succinic dehydrogenase which was used was the reconstructed succinic oxidase system of Keilin & Hartree (1938, 1940). Succinic α -D₂, α' -D₂ acid (tetradeuterosuccinic acid) was prepared, and its rate of enzymic oxidation was compared with that of normal succinic acid, prepared by analogous methods, by measurement of oxygen consumption in Warburg manometers at 37°. It was found that tetradeuterosuccinic acid was oxidized at about 40 % of the rate of oxidation of normal succinic acid. By measurement of the rates of oxidation at different temperatures it was demonstrated that the slower reaction of the deuterated

substrate was accompanied by an activation energy, higher than that of the reaction of the normal substrate by about 1 kcal. The activation energy of the reaction of succinic acid was 11.5 ± 0.15 kcal, this result is in fair agreement with the figure of 11.2 ± 0.2 kcal obtained by Hadidian & Hoagland (1939).

Furthermore, by measuring the rates of oxidation at a number of substrate concentrations, the Michaelis constants of the enzyme, calculated by the methods of Lineweaver & Burk (1934), were found to be 1.2×10^{-3} M with respect to succinic acid, and 2.2×10^{-3} M with respect to tetradeuterosuccinic acid. A difference in Michaelis constants was confirmed by competitive mixed substrate experiments in which it was shown that succinic acid in mixtures with tetradeuterosuccinic acid was oxidized preferentially.

Succinic α -D, α' -D acid (dideuterosuccinic acid) was also prepared, and was found to be oxidized at about 70 % of the rate of oxidation of normal succinic acid. This figure is in agreement with the results obtained by Erlenmeyer, Schonauer & Sullmann (1936).

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Oxidation of the Blood Group A-substance with the Periodate Ion. By D. AMINOFF and W. T. J. MORGAN (*Lister Institute of Preventive Medicine, London, S.W. 1*)

Apart from the methylation studies of Bray, Henry & Stacey (1946), few structural investigations have been carried out on the blood group A substance. Oxidation of the A-substance with periodate offers an additional method of investigation.

A rapid loss of both serological properties of the A substance results on oxidation at R.T. with periodate at pH 5 (acetate buffer) and at pH 7-7.5 (NaHCO₃). The action at pH 5 is essentially complete in 6 hr. Results indicate that one molecule of periodate is reduced by 430 g. of A-substance. At pH 7-7.5 oxidation is more extensive, and even after 74 hr. substantial reduction of the periodate is still observed.

Qualitative tests on, and chromatographic analysis of, the A substance after oxidation at pH 5

for 24 hr. indicate that of the constituents potentially susceptible to oxidation only fucose, galactose and part of the chondrosamine are oxidized.

Formaldehyde and formic acid, but no acetaldehyde or ammonia, are produced during the oxidation. The formaldehyde liberated was determined and characterized as its dimedone derivative (Reeves, 1941). Methyl-N-acetylglucosamine gives no formaldehyde, whilst N-acetylglucosamine yields 93% of the expected formaldehyde. The formic acid liberated was determined after oxidation at 2-4° with NaIO₄ for 25 hr. (Potter & Hassid, 1948). The amount of formaldehyde and formic acid formed can be used as a measure of the chain length. They are produced in equimolecular proportions and agree with a molecular weight of 1800 as obtained from the

analytical figures of the constituents of the A-substance

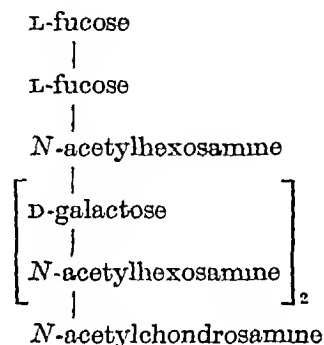
The A-substance gives a colour with Ehrlich reagent (see Morgan & Elson, 1934) equivalent to about a quarter (13 %) of the total *N*-acetylhexosamine (46 %) As methyl-*N*-acetylglucosaminide does not give a colour under these conditions it may be assumed that one in four of the *N*-acetylhexosamine units is a reducing terminal group From the chromatographic data this is *N* acetylchondrosamine, and accounts for the formaldehyde liberated

An acetic acid hydrolysate of the A-substance yields on oxidation with periodate, acetaldehyde but not ammonia Re oxidation of the acid hydrolysis products of the oxidized (pH 5.0, 24 hr) A-substance yields no acetaldehyde Baer, Dische & Kabat (1948) and Aminoff, Morgan & Watkins (1948) have shown that mild acid hydrolysis of the A-substance readily eliminates the fucose without significant liberation of the other sugars, which suggests that fucose is an end-group and, from the oxidation results, that it is a non-reducing end-group which carries a free hydroxyl group attached to C atom 4, and possesses a pyranose ring structure

Kabat, Baer & Knaub (1949) record a figure of 18%

for a preparation of our A-substance On the basis of this figure a molecular unit of 1800 would contain two fucose residues The acid liberated, however, accounts for one terminal fucose residue only Carbon atom 3 in the fucose units must be unsubstituted to account for the observation that all the fucose in the A substance is oxidized Hence, the hydroxyl group at carbon atom 2 of the non terminal fucose molecule is presumably involved in the glycosidic linkage

The following structural unit for a polysaccharide moiety of the A-substance is in agreement with the serological, qualitative and quantitative chemical data obtained



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Turbidities in the Estimation of Serum Proteins by the Biuret Method By J W KEYSER and J VAUGHN (Welsh National School of Medicine, Royal Infirmary, Cardiff)

Lipæmic sera are occasionally encountered which give a turbid or opalescent solution when treated with sodium hydroxide and copper sulphate in the biuret protein estimation Such turbidities may greatly increase the readings obtained with a photoelectric absorptiometer, and results may be up to 3 g/100 ml. too high In Kingsley's (1942) method, shaking with ether is used to eliminate turbidities An alternative procedure, which we have applied in a slight modification of Fine's (1935) method, is to treat the reaction mixture with potassium cyanide, this discharges the colour (presumably by forming the complex ion $\text{Cu}(\text{CN})_4^{4-}$) without apparently affecting the turbidity, which can then be measured

A reading is first taken on the photoelectric in-

strument using the appropriate green filter A large knife point of solid potassium cyanide (about 0.15 g for 6.5 ml of test solution) is then added with mixing and a final reading is made when the maximum discharge of colour has taken place and the colorimeter reading is constant (usually after 4-5 min) The difference between the first and final readings is used for obtaining the protein content of the specimen Of the Hilger Spekker set of colour filters, the green (no 5) and neutral grey are both suitable for the turbidity reading

This method is not applicable to turbidities that develop in the test solution on long standing, e.g. after several hours, for such turbidities may be appreciably decreased by cyanide

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The Estimation of Serum and Plasma Proteins. By J W KEYSER (*Welsh National School of Medicine, Royal Infirmary, Cardiff*)

Copper sulphate specific gravity method Comparison with the micro-Kjeldahl method shows that the copper sulphate specific gravity method of Phillips, Van Slyke, Dole, Emerson, Hamilton & Archibald (1945) gives clinically reliable results in the absence of marked change in the non-protein constituents. From over 100 persons, most of them hospital patients in whom there was no evidence of uraemia, 132 specimens of non-lipæmic serum or heparinized plasma were examined. Results by both methods differed by not more than 0.4 g/100 ml in all but two cases, by not more than 0.3 g in 84%, and by not more than 0.2 g in 56%.

Biuret method In the biuret method, considerable variations in intensity of colour development may occur if a given technique is not closely followed. Good colour development and consistency of results were found by the following modification of Fine's (1935) method. The protein precipitated from 0.2 ml of plasma or serum is dissolved in 2 ml of 3.75N-NaOH. 1 ml of 5% w/v $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ is added and mixed with the NaOH solution for 30 sec (e.g. by revolving the tube between the hands), care being taken to avoid frothing. The mixture is

allowed to stand for a further minute, then diluted to 10 ml. A bung is inserted and the contents of the tube mixed for 1 min, the precipitate finally being spun down. Occasional turbidities may be allowed for by the method of Keyser & Vaughn (1949).

Micro-Kjeldahl method In the micro-Kjeldahl method accurate results can be obtained with 0.1–0.2 ml of plasma by using a straightforward distillation apparatus consisting of a 100 ml Kjeldahl flask, splash head and double-jacketed condenser, instead of the more elaborate steam-distillation apparatus usually recommended. The ammonia is most conveniently and accurately determined by collection in boric acid followed by direct titration with mineral acid (Winkler, 1913). 5 ml of a 2% solution of boric acid is ample for collecting the ammonia formed by digestion of 0.2 ml of plasma. A suitable indicator is the B.D.H. '4.5' or Tashiro's methyl red-methylene blue mixture.

'Albumin' Kingsley's (1940) Na_2SO_4 -ether technique was compared with Howe's (1921) method using 22 specimens from 19 pathological and normal cases. Results agreed to within 0.25 g/100 ml.

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A Metabolic Product of *Aspergillus ustus* (Bainier) Thom & Church By C E STICKINGS (*Department of Biochemistry, London School of Hygiene and Tropical Medicine*)

The metabolism solution of a strain of *Aspergillus ustus*, grown on Raulin-Thom medium, gives an intense brownish purple colour with ferric chloride. Of three other strains investigated, two give similar, but less intense, colorations, while the third gives a much browner and less intense colour.

The material responsible for the purple colour reaction is extracted from the acidified metabolism solution by ethyl acetate. After evaporation, the residue is treated with ether, and a fair quantity of insoluble material is filtered. The filtrate is evaporated, and the residual gum treated with methanolic potassium acetate, when a crystalline potassium salt separates. The free acid obtained from the salt crystallizes from ethyl acetate-benzene in colourless prisms, m.p. 167°, with decomposition, yield, about 0.5 g/l of metabolism solution.

Analysis suggests the formula $\text{C}_{11}\text{H}_{12}\text{O}_7$, with one methoxyl group.

Cold neutral copper sulphate converts $\text{C}_{11}\text{H}_{12}\text{O}_7$ in high yield into an oxidation product $\text{C}_{11}\text{H}_{12}\text{O}_8$. The chemical properties of $\text{C}_{11}\text{H}_{12}\text{O}_7$ and $\text{C}_{11}\text{H}_{12}\text{O}_8$ closely resemble those of the compounds $\text{C}_{10}\text{H}_{10}\text{O}_6$ and $\text{C}_{10}\text{H}_{10}\text{O}_7$.

(I, R = $-\text{CH}(\text{OH})\text{COCH}_3$ and $-\text{C}(\text{OH})_2\text{COCH}_3$, respectively) isolated as metabolic products of *Penicillium brevi-compactum* Dierckx (Oxford & Raistrick, 1933).

$\text{C}_{11}\text{H}_{12}\text{O}_8$ is oxidized smoothly by alkaline H_2O_2 to a compound $\text{C}_9\text{H}_8\text{O}_7$, acetic acid also being produced in the reaction. Methylation of $\text{C}_9\text{H}_8\text{O}_7$ gives 3,4,6-trimethoxy-phthalic acid, identified by comparison with synthetic material. It is also identical with the

fully methylated phthalic acid obtained by ethanolic alkaline hydrolysis of *O* dimethyl citromycetin methyl ester, and subsequent methylation $C_9H_8O_7$ is therefore a methoxy-dihydroxy-phthalic acid of the same orientation

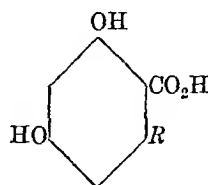
The oxidation product $C_{11}H_{12}O_8$, which behaves in many ways as $C_{11}H_{10}O_7 \cdot H_2O$, can be decarboxylated, and then oxidized by alkaline H_2O_2 to a compound $C_8H_8O_5$, which has been methylated to 2,3,5 trimethoxybenzoic acid, and ethylated to 2 methoxy 3,5 diethoxy-benzoic acid, identified by comparison with synthetic specimens prepared by the Elbs persulphate oxidation of α -resorcylic acid and subsequent alkylation

$C_8H_8O_5$ is therefore 2-methoxy 3,5-dihydroxy benzoic acid (III), and $C_{11}H_{12}O_7$ is thus shown to be II

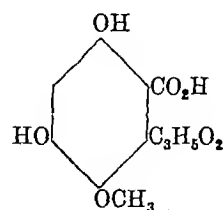
The side-chain, $-C_3H_5O_2$, should probably be formulated as the ene diol, $-C(OH)=C(OH)CH_3$, though other formulae (e.g. $-CH(OH)COCH_3$, $-COCHOHCH_3$) are possible. Oxidation by neutral cupric sulphate would be expected to give the diketone $-COCOCH_3$, further oxidation with alkaline H_2O_2 yielding a carboxylic acid and acetic

acid, as observed. Further experiments are in progress to ascertain the exact nature of this side-chain

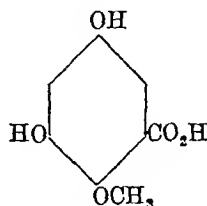
The formula IV ($R = -C(OH)=C(OH)CH_3$, $-CH(OH)COCH_3$ or $-COCHOHCH_3$) is therefore proposed for the mould metabolic product $C_{11}H_{12}O_7$



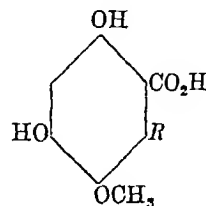
I



II



III



IV

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The Iron Requirement of Rumen Bacteria By MARY L. McNAUGHT and E. C. OWEN (*Hannah Davry Research Institute, Kirkcubbin, Ayr*)

The widespread incidence of deficiency of the metals Cu and Co in the pastures on which sheep and certain cattle habitually subsist, together with the fact that only ruminants are known to be affected by such deficiencies, has led to the hypothesis that certain metals may be required not so much by the ruminant itself as by the micro-organisms which it harbours in its gut. Tosić & Mitchell (1948), after having shown that dietary Co is concentrated in the bacteria in the rumen, suggested that perhaps these bacteria rendered Co unavailable to the host and so increased the host's apparent requirement of that metal. The relative importance of these hypotheses can be decided only by further knowledge.

Before this recent work on Co was published a series of investigations was being carried out by the present authors on the requirements of these micro-organisms for various metals, by artificially creating deficiencies in rumen liquid *in vitro* by means of organic compounds which chelate with metals, a

technique which is now much in use (Albert, Rubbo, Goldacre & Balfour, 1947; Hickey, 1945; Zentmyer, 1944).

The reagents for iron, $\alpha\alpha'$ -dipyridyl and *o*-phenanthroline at 0.001M concentration were among the chelating agents which were found to inhibit the growth *in vitro* of rumen bacteria. The inhibition was reversed by addition of an amount of $FeSO_4$ just sufficient to form the chelate complex. The minimum concentration of *o*-phenanthroline required to suppress bacterial growth, as indicated by protein synthesis, was about 0.00005M. Theoretically this amount should inactivate 0.93 p.p.m. of iron. The amount of iron in rumen liquid was found by analysis to be of this order. By completely inhibiting protein synthesis with *o*-phenanthroline and then adding various amounts of iron it was estimated that about 1.0 p.p.m. of iron is essential for bacterial growth.

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Biochemical Experiments with Radioactive 2,3-Dimercaptopropanol (BAL) By S D SIMPSON and L YOUNG* (*Department of Biochemistry, University of Toronto*)

In 1944-5, during the course of a series of investigations which were carried out in this laboratory on the antidotal action of thiols (Young, 1946), 2,3-dimercaptopropanol (BAL) containing radioactive sulphur (^{35}S) was synthesized and used to study the fate of BAL in the rat. Results in general agreement with those obtained in this work with radioactive BAL have been reported by Peters, Spray, Stocken, Colhe, Grace & Wheatley (1947).

Radioactive BAL was obtained by allowing 2,3-dibromopropanol to react in methanol with sodium hydrosulphide which had been prepared from hydrogen sulphide containing H_2^{35}S . The radioactive BAL was extracted from the reaction mixture and purified by fractional distillation at low pressure. The product weighed 0.663 g, and this represented a yield of 46% based on the amount of dihalide used. The purity of the product was 97.3% by iodine titration.

The absorption, distribution and excretion of ^{35}S were studied following the administration of radioactive BAL to rats by application to the skin or by intramuscular injection. When radioactive BAL

was applied to the skin it was absorbed slowly, and in the 6 hr period following its application the average rate of absorption was 0.38 mg BAL/sq cm skin/hr. When radioactive BAL (20 mg dissolved in 0.30 ml of propylene glycol) was injected into the muscles of a hind leg, it passed quite rapidly from the site of injection and only 2% of the ^{35}S administered as BAL was present in the dosed muscles 6 hr after the injection. Six hr after the administration of radioactive BAL by application to the skin or by intramuscular injection, ^{35}S was found distributed throughout the organism. The concentrations of ^{35}S in the kidney and in the small intestine and its contents were somewhat higher than those in other tissues, and this was probably related to the passage of radioactive material into the urine and bile. Examination of the urine showed that a large proportion of the ^{35}S which passed from the site of administration of the radioactive BAL was excreted in the urine, e.g. 72% of the ^{35}S absorbed from the skin during a 6 hr absorption period and 81% of the ^{35}S given by intramuscular injection were excreted in the urine in the 24 hr period which followed the administration of the radioactive BAL. Almost all the ^{35}S in the urine was present in the neutral sulphur fraction.

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Distribution of Nitrogenous Constituents in the Urine of Rats with Jensen Sarcoma By S J BACH (*School of Biochemistry, University of Cambridge*) and G A MAW* (*Department of Biochemistry, University College, London*)

The object of this study was to investigate the effect of one type of malignancy on the nitrogen metabolism of the tumour-bearing animal, as reflected in changes in the output of nitrogenous end-products in the urine.

The 24 hr excretions of a number of the nitrogenous constituents of the urine of groups of rats were examined immediately after inoculation with Jensen sarcoma tissue and followed during the growth of the resultant tumours for periods up to 23 days. The urinary total-N excretion of the sarcomatous rats was found in every case to be decreased relative to that of control animals. This nitrogen retention was more marked in the case of rats with large and rapidly growing tumours, being due, pre-

sumably, to satisfaction of the nitrogenous needs of the tumour itself. The urea-N and ammonia-N outputs of tumour-bearing rats, expressed as percentages of the total-N excretion, were almost identical with the corresponding data for control rats. The excretion of creatinine-N appeared to bear no consistent relation to the presence of a tumour on the rat or to the rate of growth of such a tumour. The periodic fluctuations in output were, however, much greater than in the control animals. On the other hand, the excretion of creatine-N was found to be generally increased over that of the controls, the increase becoming more marked with the development of the tumour. This is in agreement with the results of Ordway & Morris (1913), who determined the output of several nitrogenous constituents in the urine of three rats with slow growing Jensen sarcomas.

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This augmented creatinuria could be due to an increased synthesis in these animals or to a depletion of the creatine content of the tissues. The latter is considered at least a partial explanation since determinations of total carcass creatine-N in the sarcomatous rats indicated extensive losses due to a marked decrease in muscle creatine. Over periods of up to 3 weeks the carcass and muscle creatine-N values had dropped to about 50 % of the corre-

sponding values of the controls. The total N content of carcass and muscle showed a decrease of only 20 % so that the loss in creatine-N cannot be considered to reflect simply the general destruction of muscle tissue. It seems possible that this effect may be partly caused by a disturbance of the phosphorylating mechanisms in muscle under these conditions, with an increased creatinuria as one consequent symptom.

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p-Aminosalicylic Acid Estimated as *m*-Aminophenol By A L TÁRNOKY and V ANNE L BREWS (Group Laboratory, Mile End Hospital, London, E 1)

Newhouse & Klyne (1949) have recently described a method for estimating *p* aminosalicylic acid (PAS) in body fluids, using Bratton & Marshall's method for sulphonamides. In the present method PAS is decarboxylated, and a Bratton & Marshall technique is applied to the *m* aminophenol (MAP) thus formed.

0.2 ml blood in 3.2 ml water is allowed to stand for 30 min. 0.6 ml 20 % trichloroacetic acid is added, and the mixture is centrifuged. 2.0 ml supernatant liquid in a 15 ml graduated centrifuge tube are heated for 1 hr at 100° with 0.5 ml 21.5N-H₂SO₄. After cooling, adding 1.0 ml 8.1N-NaOH, and cooling again, the volume is adjusted to 4.0 ml with water. 0.2 ml 1.0 % NaNO₂ is then added with shaking, after 5 min 1.0 ml 5.0 % ammonium sulphamate is added, followed, 20 sec later, by 1.0 ml 0.1 % naphthylethylenediamine dihydrochloride. (The short sulphamate time of 20 sec has recently been adopted from Newhouse & Klyne's method, it increases colour intensity by c. 20 %.) The colour reaches its maximum intensity in 2½ hr and is stable for 90 hr. Read against a

water-naphthylethylenediamine blank, in an E E L Portable Colorimeter using an Ilford '404' green filter, the colours appear proportional at concentrations from 2.5 to 20 mg PAS/100 ml.

A stock standard of 100 mg PAS/100 ml strength is prepared by dissolving 137.9 mg sodium *p*-aminosalicylate dihydrate or 71.24 mg *m* aminophenol (with the addition of 1 ml N-HCl) in 100 ml water. Dilute standards representing 5, 10 and 20 mg PAS/100 ml blood are prepared to contain 0.25, 0.50 and 1.0 ml stock standard respectively, with 15 ml 20 % trichloroacetic acid, in 100 ml aqueous solution. These solutions are treated as the supernatant liquids, using MAP standards, heating may be omitted.

Milder forms of hydrolysis have failed to effect complete decarboxylation. Experiments with standard solutions of PAS and MAP have furnished evidence that in our conditions decarboxylation does in fact take place and is complete. They also support the theory on which this method was based, namely, that MAP is more readily diazotized than is PAS.

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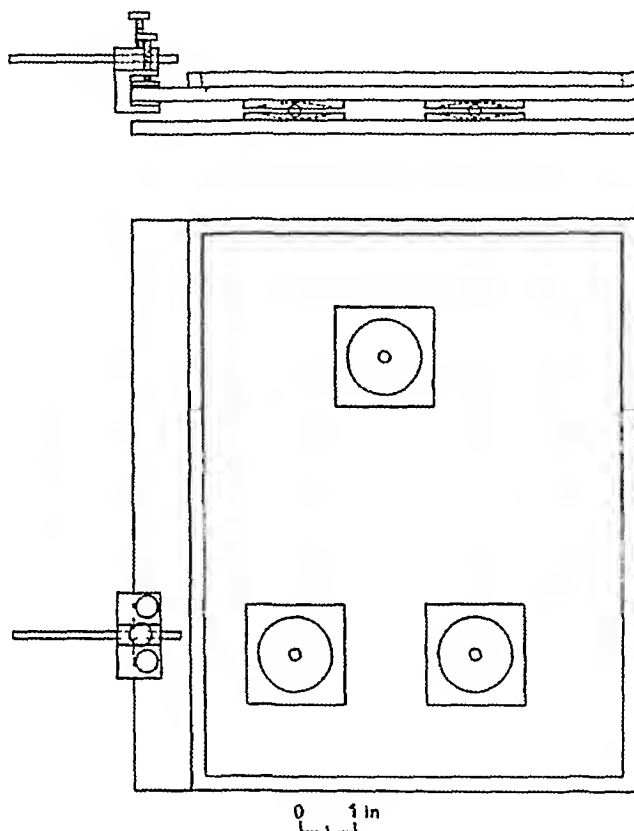
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DEMONSTRATION

A Simple Shaking Device for Conway Units and Similar Appliances By W M DALE (*Christie Hospital and Holt Radium Institute, Manchester*)

It was found useful to employ a device for the simultaneous shaking of a number of Conway Units, either by hand or geared motor

impulse from its resting position, returns to it by three-dimensional wobbling movements which persist for a considerable time and impart a



The apparatus consists of two glass plates ($\frac{1}{4}$ in thick) each mounted with three hollow-ground slides which, when coinciding, form three shallow cups to contain three steel ball-bearings of $\frac{1}{8}$ in diameter. The upper plate, when moved by an

very suitable mixing movement to Conway dishes placed on the upper plate within a wooden frame. For continuous shaking a geared-down motor provides an occasional impulse to the protruding rod.

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*

Globulin complexes with oestrogenic acids By W HAUSMANN and A E WILDER SMITH

Seed globulins of the Gramineae and Leguminosae By C E DANIELSSON

Carotenoids of loquat (*Eriobotrya japonica* Lindl) By J C SADANA

Improved separation of sugars on the paper partition chromatogram By M, A JERMYN and F A ISHERWOOD

Zinc uptake by *Neurospora* By IRMA ANDERSSON-KOTTO and G CH HEVESY

Accumulation of glutamic acid in isolated brain tissue By J R STERN, L V EGGLESTON, R HEMS and H A KREBS

Displacement chromatography on synthetic ion exchange resins 1 Separation of organic bases and amino acids using cation exchange resins By S M PARTRIDGE and R G WESTALL

Purification of alkaline phosphatase By M A M ABUL-FADL, E J KING, J ROCHE and NGUYEN-VAN THOAI

Purification of faecal alkaline phosphatase By M A M ABUL-FADL and E J KING

Purification of alkaline phosphatase by tryptic digestion By M A M ABUL-FADL and E J KING

Reactivation of alkaline phosphatases after dialysis By M A M ABUL-FADL and E J KING

The action of tyrosinase on monophenols By L P KENDAL

Polysaccharides synthesized by aerobic mesophilic spore-forming bacteria By W G C FORSYTH and D M WEBLEY

The pathway of the adaptive fermentation of galactose by yeast By J F WILKINSON

Studies on the lens By M LANGHAM and H DAVSON

Breakdown of cozymase by a system from nervous tissue By H McILWAIN and R RODNIGHT

The oxidation of catechol and homocatechol by tyrosinase in the presence of amino acids By H JACKSON and L P KENDAL

β Glucuronidase as an index of growth in the uterus and other organs By LYND A M H KERR, J G CAMPBELL and G A LEVY

Concentration of lipids in the brain of infants and adults By A C JOHNSON, A R McNABB and R J ROSSITER

Effects of amidines on oxidases of *Escherichia coli* and of animal tissues By J J GORDON and ENID SOWDEN

The quantitative determination of barbiturates in tissues by ultraviolet absorption spectrophotometry By G V R BORN

Nicotinamide biosynthesis by intestinal bacteria as influenced by methyltryptophans By P ELLINGER and M M ABDEL KADER

The linkage of glutamic acid in protein molecules By F HAUROWITZ and F BURSA

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The Annual General Meeting of the Biochemical Society was held in the Department of Biochemistry, University College, Gower Street, London, W C 1, on Friday, 25 March 1949, when the following papers were read

COMMUNICATIONS

The Effects of Thyroxine and Thio-uracil on the Secretion of the Phosphorus Compounds Normally Present in Milk By R. CHANDA and E. C. OWEN (*The Hannah Davry Research Institute, Kirkhall, Ayr*)

Cows treated with thyroxine show an increase of total phosphorus in the milk without any change in calcium (Owen, 1948). Houston, Kon & Thompson (1940) found that vitamin B₁ pyrophosphate in milk varies inversely with phosphatase. The experiments described in this abstract were designed to find out what compounds of phosphorus are chiefly responsible for the increase of phosphorus in the milk of cows treated with thyroxine and also to find out whether thio-uracil can effect the composition of the milk in the opposite way to thyroxine. Some cows were injected with 10 mg thyroxine per day, while others received 20 mg thio-uracil per day, the injections lasting 3 weeks. Untreated cows served as controls. Milk samples were collected every 2 days, for 3 weeks prior to treatment (period 1), during treatment (period 2), and for 3 weeks subsequent to treatment (period 3). The partition of phosphorus and the content of phosphatase were determined in these samples. The average results of one such experiment are shown in the table.

From the table it can be concluded that the chief

contribution to the increase of phosphorus is made by esters of phosphoric acid, though a notable contribution is also made by lipid-P (see period 2 in the table). Ester-P and lipid-P steadily increased during the 3 weeks of injection of thyroxine, while phosphatase steadily declined, so that its average in period 2 was much less than it had been in period 1. When thyroxine was discontinued ester-P and lipid-P rapidly declined while phosphatase rapidly increased. All these effects took place in the reverse order in cows treated with thio-uracil (see table), while none of them was observable in untreated cows. In all cows, including the controls, there were highly significant inverse correlations between phosphatase and ester-P, amounting in treated cows to inverse proportionality. Lipid-P was inversely correlated with phosphatase during periods 2 and 3 only. The correlations between phosphatase and lipid-P in the control cows and in period 1 in all the cows were not significant. The bearing of these results on mammary secretion will be discussed when the work is published in full.

Partition of phosphorus (mg/100 g milk)

Animal	Period	Total P	Inorganic P	Ester P	Lipoid-P	Casein P	Phosphatase
Dinky (control)	1	86.65	55.37	7.68	6.2	17.40	112
	2	86.00	56.67	7.34	6.2	16.30	121
	3	90.55	60.41	5.81	6.7	17.60	129
Dorothy (thyroxine)	1	82.41	46.45	8.41	9.3	18.10	129
	2	92.93	47.84	16.28	11.2	19.20	43
	3	84.59	47.28	9.73	9.7	18.30	120
Gadfly (thyroxine)	1	88.25	57.08	8.66	7.6	15.61	148
	2	94.51	54.19	13.84	9.7	17.18	64
	3	91.84	58.96	8.85	8.6	15.42	125
Misty Morn (thio uracil)	1	94.40	59.60	8.97	8.3	17.81	129
	2	85.13	57.74	5.15	6.9	15.51	213
	3	93.05	62.54	6.18	8.4	16.38	165
Trixie (thio uracil)	1	98.74	60.97	9.90	9.2	18.50	138
	2	91.96	61.28	5.03	7.1	18.20	209
	3	98.40	65.11	6.96	9.1	17.00	170

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Glucuronide Synthesis and Cell Proliferation By M C KARUNAIRATNAM, LYND A M H KERR and G A LEVY (Department of Biochemistry, University of Edinburgh)

In view of the relationship found to exist between the state of proliferation and the β glucuronidase activity of mouse liver, kidney and uterus (Levy, Kerr & Campbell, 1948, Kerr, Campbell & Levy, 1949), the possibility that the ability of mouse liver and other tissues to synthesize glucuronides may also vary with the degree of cell division in progress was investigated by the method of Levy & Storey (1949)

Unlike β glucuronidase activity, the ability to synthesize glucuronides seemed to be confined largely to liver. Kidney, the only other tissue found to carry out the synthesis, was much less active than liver. This is in agreement with the results of Lipschitz & Bueding (1939)

During the period of enhanced glucuronidase activity in liver which follows partial hepatectomy or administration of carbon tetrachloride or menthol, the ability to synthesize glucuronides remained

normal. It was also unchanged during the profound depression of glucuronidase activity which follows sorbic acid administration. Tumours with high glucuronidase activity were devoid of synthetic power, as were livers from mice 2–3 days old. It seems clear that, unlike β glucuronidase, the enzyme system causing glucuronide synthesis does not vary in activity with the state of proliferation of a tissue, and that the two enzyme systems are quite distinct (see Levy, 1948, Karunairatnam & Levy, 1949). The synthetic power of mouse liver remained very small for the first week after birth. It then rose slowly till at the fourth week the activity had reached the average value for normal adults. The β glucuronidase activity of liver in the first week after birth was six times the value found in adults. It then fell steadily to become constant when the mice were 4 weeks old.

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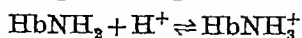
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The Carbamino Combination of CO₂ with Reduced Haemoglobin and Oxyhaemoglobin By F J W ROUGHTON (Department of Colloid Science, University of Cambridge)

Wyman (1948) has recently suggested that carbamino combination of CO₂ with haemoglobin only occurs with —NH₂ groups of pK \gg 8.0. He thence deduces the approximate equation

$$Z = K_1 K_2 p \text{CO}_2 / [\text{H}^+]^2, \quad (1)$$

where $Z = [\text{carbamino CO}_2] / [\text{O}_2 \text{ capacity of Hb}]$
 K_1, K_2 are the respective equilibrium constants of



and $\text{HbNHCOO}^- + \text{H}^+ \rightleftharpoons \text{HbNH}_2 + \text{CO}_2$

Equation (1) can be tested by the data on (a) ox Hb (Ferguson & Roughton, 1934), (b) human Hb (Ferguson, 1936), (c) horse Hb (Stadie & O'Brien,

1937). In all three papers, Z , $p\text{CO}_2$ and total CO₂ were determined directly, but only in (c) was the pH measured directly (glass electrode). In this case calculations show (both for O₂Hb and RedHb) that

$$\text{pH} = 6.17 + \log \{ [\text{total CO}_2] - [\text{carbamino CO}_2] - [\text{dissolved CO}_2] \} / [\text{dissolved CO}_2] \quad (2)$$

In (a) and (b) the pH can reasonably be calculated from (2)

I give below calculations of $K_1 K_2 \times 10^{17}$ for all the reliable data cited in (a), (b) and (c). Figures in brackets denote the number of experiments on each sample of Hb

(a)*		(b)		(c)	
RedHb	O ₂ Hb	RedHb	O ₂ Hb	RedHb	O ₂ Hb
1.1 (1)	0.40 (1)	1.33 (4)	0.44 (2)	0.97 (4)	0.67 (6)
0.94 (3)	0.48 (3)	1.1 (4)	0.74 (4)		
1.06 (3)	0.54 (3)	1.0 (1)	0.40 (1)		
1.00 (4)	0.49 (2)	1.81 (1)	1.25 (1)		
1.14 (2)	0.54 (2)	1.58 (3)	0.59 (3)		
Mean of all determinations	1.03	1.33	0.66		

* Most of the (a) results were plotted graphically, but reference to the original notes gave all the data required for the calculations

Both the (a) and the (b) data show the same average ratio of K_1K_2 for RedHb to K_1K_2 for O_2 Hb, i.e. 2.0, the (b) data are, however, much more scattered, perhaps because carbamino estimations are technically less satisfactory in human than in ox Hb. Unfortunately, only one Hb sample was used in (c).

The present results fail to sustain Wyman's views that (i) the $-NH_2$ groups with which CO_2 combines

are unaffected by oxygenation of the Hb molecule, (ii) the extra carbamate in RedHb over that found in O_2 Hb is entirely due to the pH being more alkaline in the former case, (iii) carbamino compounds play much less part in blood transport of CO_2 than currently supposed. Wyman's views, which were largely based on more limited and less direct calculations than those here given, are open to other criticisms, which it is hoped to develop elsewhere.

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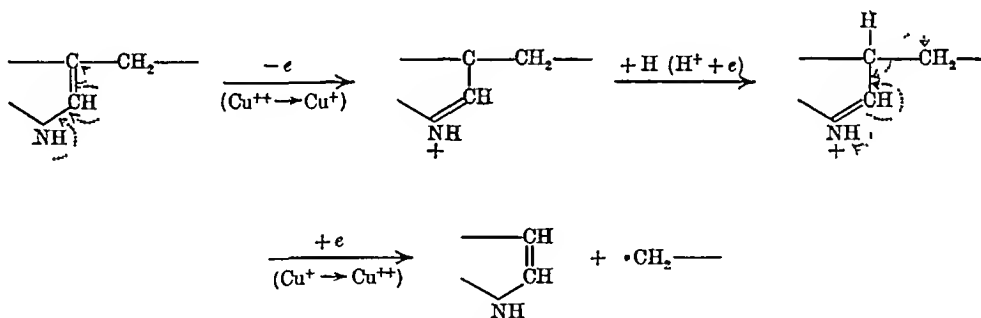
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Metal Catalysis of Indole Production from Tryptophan By J. W. BAKER, E. A. DAWES and F. C. HAPPOLD

Seeking to elucidate the mechanism of the production of indole from tryptophan by the tryptophanase system of *Esch. coli*, a reaction unique in enzyme chemistry inasmuch as fission of a C_3-C_β bond occurs, we have investigated related inorganic catalyses of tryptophan decomposition.

cobalt, iron, manganese, nickel, chromium, tin and cerium, but of these only cobalt showed any catalytic effect, the magnitude of which is approximately of the same order as that of copper, and which is detectable down to concentrations of $5 \times 10^{-6}M$. The mechanism of the metal-catalyzed reaction is not



(Dotted arrows denote single electron transfers.)

Herzfeld (1913) obtained a 60% yield of indole from tryptophan in the presence of $CuSO_4$ and 9% (w/v) NaOH. We have verified his results and extended investigations to other metals. The rate of indole production was followed by determinations on successive 10 ml. fractions of distillate or on fractions of distillate collected over 15 min. periods. Slight indole formation was found to occur in the presence of NaOH only, due to trace metals present in the glassware, but greatly increased production occurred on addition of $CuSO_4$.

Other metals were also investigated, including

identical with that of the enzymic reaction, since other indole derivatives such as indolyl-3-glycine and β -indolyl-3-ethylamine (which do not give indole with tryptophanase) are degraded to indole by the alkaline copper and cobalt reagents. Analogy with the catalytic effects of these metals in other chemical reactions suggests that a single-electron transfer mechanism, of the general type given above, might be applicable to the reaction, although the presence of free radicals could not be detected by the use of methyl methacrylate monomer or 'luminol'.

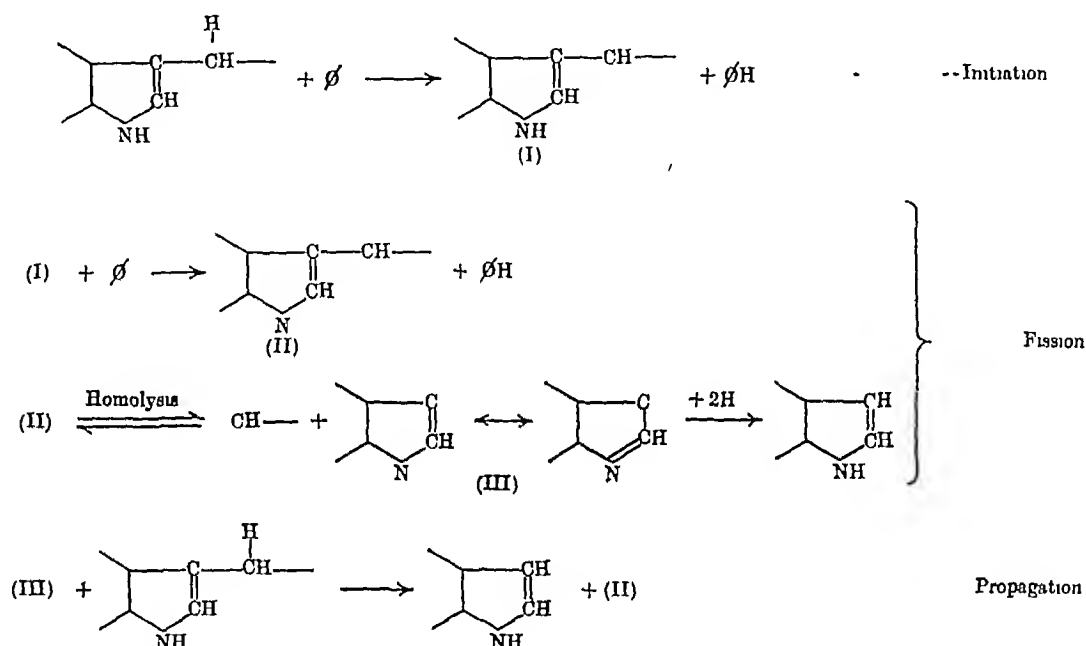
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Suggested Mechanism for the Enzymic Breakdown of Tryptophan to Indole By J W BAKER,
E A DAWES and F C HAPFOLD

Subsequent investigations have confirmed the earlier conclusions of Baker & Hapfold (1940) and Baker, Hapfold & Walker (1946) that, in the enzymic formation of indole from tryptophan, the three-carbon side-chain is eliminated intact (see also Tatum & Bonner, 1944, Dawes, Dawson & Hapfold, 1947, Wood, Gunsalus & Umbreit, 1947), and that indole formation must occur by the *reductive-fission* of the $\geq C_\beta-C_\alpha$ bond to give $\geq CH + \geq CH$. The

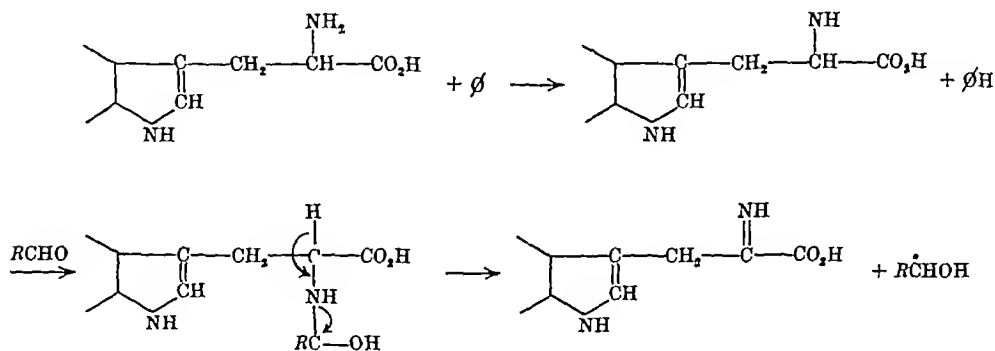
phan by mechanisms involving single electron transfers. Moreover, Waters (1946) has quoted evidence that free radicals may be generated in metal-free biological systems. Accordingly, a tentative elaboration of Baker & Hapfold's original mechanism for the enzymic fission, based on enzyme-produced free radical (ϕ) initiation, and with the coenzyme, pyridoxal phosphate, acting as the hydrogen acceptor, is formulated below



only known mechanism for such rupture is via an initial homolytic fission to give free radicals $\geq C + C\leq$, stabilized by resonance. Although the mechanism of the metal-catalyzed fission of tryptophan to indole (previous abstract) must differ somewhat from the enzymic fission, it provides strong evidence that indole can be produced from trypto-

The large resonance stabilization of the radical (III) would lower the energy necessary for the homolytic fission of the $C_\alpha-C_\beta$ bond.

Free radical dehydrogenation at C_α or $\alpha-NH_2$ in the side chain would yield α -imino- β -3-indolylpropionic acid, the precursor of indole-pyruvic acid fission, observed with the purified enzyme system.



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Serum Flocculation Tests at pH 7.55 By A. L. LATNER and G. B. PENDLENTON

The thymol buffer used by MacLagan is stated by him to have a pH of 7.8 (MacLagan, 1944). He also stresses the importance of ionic strength. American observers have claimed that the reaction is more sensitive at pH 7.55 (Mateer, Baltz, Commanduras, Steele & Brouwer, 1947). It has been pointed out, however, that they apparently had an undue proportion of negative results (MacLagan, 1947).

Thymol buffers at pH 7.8 and 7.55 have been compared, the pH was determined by the bicolor technique (Hastings & Sendroy, 1924). The ionic strength in each case approximated fairly closely to that of MacLagan's buffer. That of pH 7.55 had the

composition 1.518 g of barbitone and 0.927 g of sodium barbitone per 500 ml saturated thymol solution. Both buffers were prepared according to MacLagan's technique.

At pH 7.55 the reaction was more sensitive and gave fewer false positives than at pH 7.8. It also proved superior to MacLagan's buffer which by the bicolor technique was found to have a pH of 7.65.

Similar results were obtained with the colloidal gold reagent. For this to have a pH of 7.55 the buffer composition is 0.6072 g barbitone, 0.3708 g sodium barbitone and 0.2 g phenol in 100 ml distilled water.

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Cozymase Degradation and the Control of Carbohydrate Metabolism in Brain By H. McILWAIN
(Research Laboratory, Maudsley Hospital, London, S.E. 5)

In animals, breathing air, physiological experiments show that the brain converts to lactic acid about 15% of the glucose which it utilizes (Gibbs, Lennox, Nims & Gibbs, 1942). This proportion increases considerably in hypoxia and in certain other conditions. In brain tissue slices, similarly, glycolysis is partly suppressed in air (the Pasteur effect). This suppression, which is of considerable importance in cellular economy, has now been found to be disturbed by agents which inhibit the breakdown of cozymase.

(1) The Pasteur effect in brain is known to be inhibited by low concentrations ($c 10^{-5}M$) of certain dyestuffs, notably phenosafranine, ethyl red and p-nitrophenol (Dickens, 1936) and by nicotine (Baker, Fazekas & Himwich, 1938). These compounds have now been found to inhibit also the breakdown of cozymase by washed, ground brain tissue. Phenosafranine was examined in detail and the breakdown (see McIlwain & Rodnight, 1948) found to be inhibited 50% at a concentration of $c 3 \times 10^{-5}M$. (In

comparing this concentration with the $10^{-5}M$ quoted above, it must be noted that the dyestuff is concentrated by the tissue to a large extent.) In contrast to this inhibition by phenosafranine, some 30 other compounds with pyridine, piperidine, phenazine and thiazine nuclei had much smaller effects, if any, on the cozymase breakdown, some of these substances are known also to have no action on the Pasteur effect (Dickens, 1936, Dickens & McIlwain, 1938).

(2) Among pyridine derivatives certain correlations have been found between structure and inhibition of cozymase breakdown, and 5(4)-3'-pyridylglyoxaline is a fairly potent inhibitor of the reaction (80% inhibition at $8 \times 10^{-4}M$, nicotinamide gives this effect at $c 8 \times 10^{-3}M$). The pyridylglyoxaline, also, at $3 \times 10^{-3}M$, releases aerobic glycolysis by brain slices, nicotinamide has a very much less effect.

(3) The pyridylglyoxaline (like nicotinamide, but at lower concentrations) maintains the anaerobic

glycolysis of brain at levels from which it would otherwise fall

(4) Nicotinamide and the pyridylglyoxaline have no effect on respiration of brain slices in concentrations at which they release or maintain aerobic or anaerobic glycolysis

(5) The enzyme degrading cozymase, in ground brain preparations and in solution after extraction and some purification, also inactivates coenzyme II with liberation of nicotinamide, but with a velocity a little lower than that of its reaction with cozymase. When both coenzymes I and II are present, com-

petition for the enzyme takes place between the two

(6) Dihydrocoenzymes I and II are acted on by the enzyme (attached to cell debris or in solution), if at all, with velocities less than 0.5% of those with which their oxidized forms are attacked

In conjunction with Judah & Williams-Ashman's (1949) observations, it would appear that the enzyme degrading cozymase functions in oxidative phosphorylations and its inhibition leaves more inorganic phosphate and cozymase available for glycolysis, than would otherwise be the case

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The Properties of β -Glucuronidase. By G. T. MILLS and J. PAUL (*Biochemistry Department, University of Glasgow*)

It was recently shown (Mills, 1948) that aqueous extracts of ox spleen when examined by the Variable Solvent Solubility Test (Falconer & Taylor, 1946), using ammonium sulphate as precipitant and L-menthyl- β -D-glucuronide as assay material, showed the presence of two β -glucuronidases. These two enzymes were distinguished by having different pH optima.

This work when repeated using phenolphthalein- β -D-glucuronide as assay material showed the same results. When phenyl- β -D-glucuronide was used as substrate, however, it was found that in addition to the two enzymes with pH optima at 4.5 and 5.2, the variable solvent solubility test showed the presence of a third β -glucuronidase having a pH optimum around 3.4 in acetate buffer. This third enzyme does not hydrolyse L-menthyl and phenolphthalein- β -D-glucuronides, and is inhibited in citrate buffer.

Citrate also shows some inhibiting action on the enzyme with pH optimum at 4.5, but exerts little if

any action on the enzyme with optimum at pH 5.2. It is significant to note in this connexion that Levvy and his co-workers (Kerr, Graham & Levvy, 1948; Levvy, Kerr & Campbell, 1948; Karunaratnam & Levvy, 1948) have used phenyl- β -D-glucuronide in citrate buffer for the assay of their glucuronidase preparations.

In addition to citrate, a number of di-carboxylic acids and poly-hydroxy-mono- and di-carboxylic acids act as inhibitors for this series of enzymes, some being much more powerful than citrate, the most powerful so far found being D-saccharate, in confirmation of Karunaratnam & Levvy (1948). Its inhibitory action is, however, more powerful than these workers suggest.

Other organs have been examined, namely, liver, kidney, uterus, thymus and blood plasma. In all cases there appear to be more than one β -glucuronidase present. Some data concerning the glucuronidases in these tissues is presented.

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Metabolism of Radioactive Strontium in the Rabbit. By MARGARET TUTT and JANET VAUGHAN
(introduced by H. BLASCHKO) (*Department of Pharmacology, University of Oxford*)

The experiments described form part of an investigation of the metabolism of radioactive strontium in normal rabbits. The animals were on a diet giving an average intake of calcium, namely, 3–5 gr in a 9 day period.

The radioactive material was a mixture of Sr^{86} and Sr^{90} as strontium chloride. It was given intravenously in normal saline, both with and without added stable strontium chloride.

The urine and faeces were collected separately, and the amount of strontium in them estimated from the time of injection until the animal was killed, on the ninth day. The strontium remaining in the body was then estimated.

The faeces were ashed in a muffle furnace, and the strontium in the dry ash measured with a G. M. no. 2 counter. The urine and bones, and samples of organs, skin and flesh, were measured in solution using an M. R. C. type 1 liquid counter.

The strontium was retained almost exclusively in the skeleton, less than 1 % being recovered from the rest of the carcass. The total retention varied with the age of the rabbit, being about 55 % in 6-week-old rabbits, 20 % in 6-month-old rabbits and 10 % in older animals.

The total excretion varied from 40 % in 6-week-old to 70–80 % in 6-month and older rabbits. The urinary excretion was greater than the faecal excretion except in the case of one 7-year-old rabbit. The excretion reached a maximum during the first 24 hr, and attained a constant low level by the fourth day.

A considerable proportion of the radioactive strontium injected into suckling mothers 1 week after delivery was transferred through the milk to the sucklings, and the percentage retained by the mothers was lower than that retained by non-suckling animals of the same age. An average of 20 % was recovered from the litters of 6-month suckling mothers, who themselves retained only 10 % of the injected dose.

On the other hand, only 1–3 % was recovered from the foetuses of pregnant mothers who were injected 9 days before the expected date of delivery and killed on that day. The percentage retained by the pregnant mothers was not appreciably lower than the average figure for the same age group.

Work has also begun on two further groups of rabbits, one of which is receiving a calcium-deficient diet, and the other a higher than normal calcium allowance.

Colorimetric Determination of Picric Acid in Picrates By R. STOHR (*Medizinisch-chemisches Institut, Universität Innsbruck, Austria*)

In the colorimetric determination of blood sugar by Benedict (1918) small quantities of glucose are brought into reaction with an excess of picric acid (reduction of picric acid to picramic acid). Under inverted proportions of the concentration (small quantities of picric acid and excess of glucose) a colorimetric method for the determination of picric acid has been worked out, which in consequence allows the determination of the molecular weight of the equivalent weight of organic basic substances in picrates.

Under the altered conditions the reduction of picric acid can possibly lead over the picramic acid as far as to the formation of diammonitrophenol. For the determination of picric acid therefore two empirical curves with picric acid solutions of known concentration have been worked out in the presence of 30 mg of glucose under certain conditions using solutions of 20 % sodium carbonate or 1 % sodium hydroxide (height of the standard 20 mm). 0.5–2.5 mg picric acid proved to be the most convenient concentration.

Procedure Quantities of picrates, corresponding to 0.5–2.5 mg picric acid, are dissolved in a 12.5 ml marked test-tube in 1 ml of 20 % anhydrous sodium carbonate or 1 % sodium hydroxide. Then 5 ml of 0.600 % glucose solution (equal to 30 mg glucose) and water are added to make it a total volume of 11 ml. After this the solution is heated in a boiling water bath for 10 min, cooled and filled up to the mark of 12.5 ml. Reading is made in the colorimeter against a standard solution prepared in the following way: 5 ml of 0.600 % glucose solution (equal to 30 mg glucose), 5 ml of water and 1 ml of 20 % sodium carbonate or 1 ml of 1 % sodium hydroxide are heated in a 12.5 ml marked test tube in a boiling water bath for 10 min. After cooling 1 ml of 0.100 % picramic acid (Egerer, 1918) is added and filled with water up to the mark of 12.5 ml. A Klett colorimeter has been used, height of the standard 20 mm.

The method enables the determination of the molecular weight of organic basic substances in picrates of known constitution, whereas in picrates of unknown constitution the equivalent weight can be determined.

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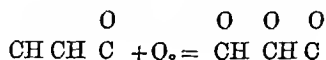
Autoxidation of the Fatty Acids III By G W ELLIS (*St Mary's Hospital Medical School, London, W 2*)

It has been possible in recent work on the oxygen uptake of mon ene fatty acids to effect the autoxidation at little above body temperatures. The products obtained are very different from those of higher temperature processes, and they may present some biochemical significance.

The dispersing medium sand, as used formerly, has been replaced by fluted strips of filter paper, either folded into a drum and placed in the autoxidizing chamber to be heated externally, or made to line the inside of the chamber and heated internally by means of an electric lamp. The chamber contains alkali to keep the incoming oxygen free from the large volumes of carbon dioxide evolved during the process. The R Q of these autoxidations is about 0.2, rising to about 0.4 and then representing with the water given off some 60 % of the oxygen uptake.

The coloured oils and resins, the scission products suberic, azelaic, octoic and nonoic acids, together with the monoacyl derivatives of the epoxy-acid, will have disappeared or been greatly reduced in yields, as a result of the lower temperatures employed. In their place may be as much as 40 % of a mixture of the 8- and 11-keto-acids. These active olefine ketone acids appear to be the initial products of autoxidation, and they are undoubtedly the substances which in the higher temperature autoxidations give rise to most of the coloured oily products.

These olefine ketones have been demonstrated as peroxide-forming. The type of structure thereby formed is postulated as that of a 6 membered heterocyclic ring. Its ready formation at room temperatures is regarded as due to the conjugated system arising as a result of the carbonyl formation and to the break of the double bond of this group.



The unstable peroxide, or ozonide, formed is regarded as readily yielding two active atoms of oxygen. The recovery of the olefine ketone may be brought about by the end of the fatty acid chain acting as oxygen acceptor, and by the tendency for recovery of the double bond of the original carbonyl group. This latter rearrangement would replace the original ethenoid bond and detach the oxygen-carbon linkage. Other peroxide structures suggested from time to time and the hydroperoxide found by Criegee, Pilz & Flygare (1939), by Farmer & Sundralingam (1942) and by Farmer & Sutton (1943) in the same relative positions to the ethenoid bond of different mon-enes, present some difficulty in explaining their function as reversible oxidative catalysts.

The olefine ketone acids are regarded as the initial autoxidation products, formed slowly by gaseous oxygen during the induction period or more readily as a result of catalysis. The subsequent rapid stage of autoxidation must be regarded as due to the peroxide formation suggested. The peroxidation products include elaidic acid epoxide, as found in appreciable amounts from both elaidic and oleic acids (Ellis, 1936), the scission products mentioned above, and the β -ketonic acids with chain degradation products, including the carbon dioxide and water observed. Evidence for the formation of these products has been obtained. In connexion with problems in biochemical oxidations, it is of interest to observe that the form of peroxide postulated, having, as it would appear, two available oxygen atoms, would not allow of formation of an intermediate β -hydroxy-acid accompanying the process of β -oxidation.

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Synthesis of Fatty Acids and Cholesterol in the Rabbit Foetus Studied with the Aid of Deuterium

By G. POPJÁK and MARIE-LOUISE BEECKMANS (*National Institute for Medical Research, London*)

In a previous investigation (Popják, 1947) it was found that all foetal tissues are able to synthesize phospholipins and that the rate of synthesis in the foetus is greater than in the mother. Results obtained in cholesterol-fed rabbits (Popják, 1946), in which the placenta was partially blocked by

accumulated cholesterol, strongly suggested that both cholesterol and fatty acids also are synthesized in the foetus and are not derived from the mother by placental transmission. The present investigation, carried out with the aid of deuterium, proves this thesis, although the results do not exclude the

possibility that some fat passes through the placenta from mother to foetus

Pregnant rabbits were given D_2O by injection and in drinking water, so as to maintain the concentration of D_2O in the body water at about 1.5% for 1-12 days. On the 28th day of pregnancy the animals were anaesthetized and their foetuses removed from the uterus. Foetal blood was drawn from the umbilical vein and maternal from the aorta. The deuterium contents of cholesterol and fatty acids, derived from phospholipids and from neutral fats and extracted from maternal and foetal tissues, were determined. In all experiments the fatty acids and cholesterol from the foetuses were richer in the isotope than the maternal compounds, indicating

a higher rate of synthesis in the foetal than in the maternal tissues, e.g. maternal liver. The foetal liver is especially active in the synthesis of cholesterol, the extra-hepatic foetal tissues (presumably the developing adipose tissue), on the other hand, are more active than foetal liver in the production of fatty acids. While the rate of renewal of both fatty acids and of cholesterol is slightly faster in the foetal placenta than in the maternal liver, it is slower than in the foetus, thus excludes the possibility that the foetal fats, rich in isotope, were transferred from the placenta.

The results further show that the rate of regeneration of cholesterol in the liver is much faster than that of fatty acids.

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The Utilization of Acetate by the Foetus for Fatty Acid and Cholesterol Synthesis By G POPJÁK and MARIE-LOUISE BEECKMANS (*National Institute for Medical Research, London*)

It is known from the work of Bloch & Rittenberg (1942) that acetate, or an active derivative of it, is utilized for both cholesterol and fatty acid synthesis (see also Bloch, 1947). We have injected intravenously pregnant rabbits, which have also received heavy water, with $CH_3^{14}COONa$ and have measured the radioactivity of the foetal and maternal fatty acids and cholesterol. The total injected dose was $50 \mu c$ of ^{14}C , in one experiment this was incorporated in 1.8 g of CH_3COONa , in others in 4.3 mg of the anhydrous salt. Three injections were given daily for 1-4 days.

The results obtained with ^{14}C labelling confirmed those obtained with the aid of deuterium (see preceding communication) and showed the utilization of acetate (or at least of the carboxyl-C of acetate) for cholesterol and fatty acid synthesis in the foetus. Also it was possible to compare the radioactivity of maternal and foetal plasma cholesterols with the radioactivity of cholesterol obtained from tissues, the activities in increasing order were: maternal plasma < maternal liver < foetal placenta < foetal carcass < foetal plasma < foetal liver. The results

strongly suggest that the primary site of plasma cholesterol synthesis, both in the mother and in the foetus, is the liver, although in the mother cholesterol with the highest ^{14}C content (approaching that of foetal liver cholesterol) was found in the intestine.

The neutral fat fatty acids of the mammary gland of the mother are synthesized at a very rapid rate. The deuterium content of these fatty acids indicated that they are synthesized at about the same rate as the foetal carcass neutral fats, but their ^{14}C content indicated a much higher rate of acetate utilization for fatty acid synthesis in the mammary gland than in the foetus or anywhere else. This observation is in excellent agreement with Folley & French's (1948) postulate of acetate utilization for fatty acid synthesis in the mammary gland of ruminants. It is noteworthy that both the D and ^{14}C contents of phospholipid fatty acids extracted from the mammary gland were much less than those of neutral fat fatty acids, whereas in other organs usually the reverse is true.

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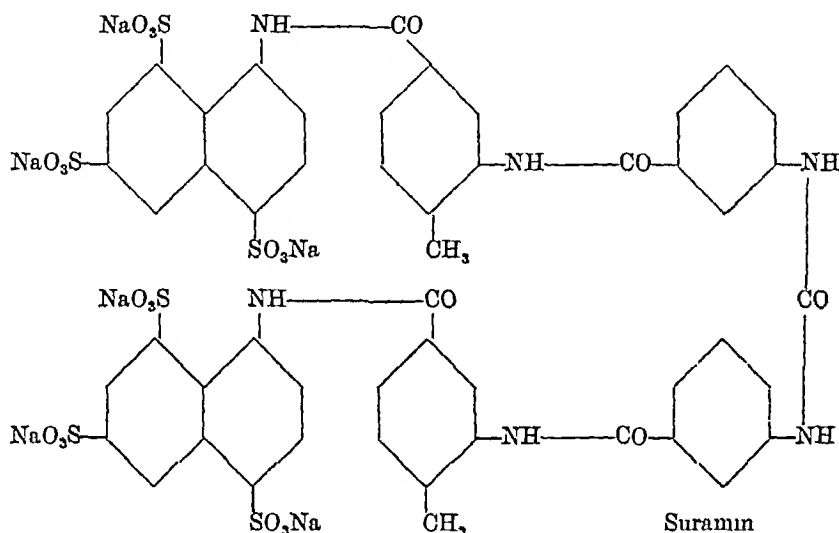
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The Action of Suramin on Proteolytic Enzymes. By B W TOWN and A WORMALL

The mode of action of suramin, a drug which has feeble trypanocidal action *in vitro* but which is very effective *in vivo*, is not known. Suramin readily combines with various proteins (Boursnell & Wormall, 1939, Dewey & Wormall, 1946), and there is also evidence that it can inhibit certain enzymes. Since it is possible that the *in vivo* trypanocidal action of the drug may be partly or wholly due to interference with enzyme systems inside the trypanosome, we have been studying the *in vitro* action of suramin on a variety of enzymes as a prelude to investigations with trypanosomes.

The suramin concentrations used are within the range of the plasma suramin level maintained for some time following the intravenous injection of a 'normal' dose of the drug, for example, the intravenous injection into rabbits of 28 mg of suramin/kg body wt (equivalent to 1.4 g/50 kg or 2.0 g/70 kg man) is followed by the maintenance, on the average, of a plasma suramin level of above $M/2000$ for 4 min, above $M/3000$ for 25 min, above $M/5000$ for 80 min, and above $M/10,000$ for 7 hr (Wilson & Wormall, 1949). Beilinson (1929) noted the inhibitory effect of suramin on trypsin, but the concentrations he



The hydrolysis of casein by trypsin is strongly inhibited by small amounts of suramin. Thus $M/2000$ suramin gives 20% inhibition of the digestion of casein by 'trypsin' (Harrington Bros Ltd) at pH 8.9 and 30° (Table 1). Even stronger inhibition was noted with Liquor Trypsin Co (Allen and Hanburys Ltd), and $M/10,000$ suramin effected 56% inhibition of the digestion of 0.36% casein solutions. Variation of the concentration of substrate with fixed concentrations of suramin and enzyme showed that, under these conditions, maximum inhibition occurred with lowest substrate concentrations (Table 2).

Table 1 Effect of suramin on the digestion of casein by trypsin at pH 8.9 and 30°

Enzyme preparation	Suramin conc	Substrate conc (%)	Inhibition (%)
'Trypsin'	$M/960$	3.65	30
	$M/1920$	3.65	20
'Liq Trypsin Co'	$M/10,000$	0.36	56

used, $M/57$ – $M/143$, are very considerably greater than any reached in the blood even after several injections of the drug.

Table 2 Inhibition of trypsin by suramin with varying concentrations of substrate

('Liq Trypsin Co' acting on casein at pH 7 and 30°, $M/2000$ suramin in all mixtures)

Casein conc (g/100 ml)	Inhibition (%)
3.65	38
1.82	45
0.91	63
0.36	83

The inhibition of tryptic action may be due to combination of suramin with the enzyme or the substrate or both, but it is of interest to note that the drug does not inhibit pepsin at pH 1–2. The possible significance of these results and their bearing on the action of suramin on trypanosomes, were briefly discussed.

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The Action of Suramin on some Non-proteolytic Enzymes By E D WILLS and A WORMALL

Suramin strongly inhibits the action of soya bean urease at pH 5.0, marked inhibition being exerted by concentrations of suramin as low as M/15,000 (Table 1). At pH 7.0, however, no inhibition occurs even with M/1000 suramin, this latter observation confirms that of Quastel (1931). Our results suggest that the drug only inhibits urease on the acid side of the isoelectric point of the enzyme (about pH 5.1). The inhibition appears to be competitive, and it is reversible, thus the enzyme 'inactivated' by suramin at pH 5.0 regains its full activity when the reaction is brought to pH 5.5.

Table 1 *Effect of suramin on urease*(Manometric method, 2.3×10^{-3} M urea, 37°)

pH	Conc of suramin	Inhibition (%)
5.0	M/3000	90
	M/5000	65
	M/15,000	25
5.3	M/1000	Nil

Trypanosomes rapidly metabolize glucose, and they appear to require carbohydrate for their survival. We have, therefore, made a special study of the action of suramin on the enzyme systems responsible for the metabolism of glucose. It is of interest to note that Marshall (1948) has recently found that trivalent arsenicals and straight-chain diamidines appear to inhibit certain enzyme systems of *Trypanosoma evansi*.

Fermentation and respiration by the intact yeast cells are not affected by suramin, almost certainly because the drug cannot penetrate the cell wall. Fermentation of glucose by yeast juice at pH 6.0 or 7.0 is, however, completely inhibited by concentrations of suramin as low as M/30,000 or sometimes M/50,000, the autofermentation of glycogen by yeast juice is also inhibited, but not quite so strongly. Of the individual yeast enzymes so far studied, hexokinase (at pH 6.0 or 7.0) is the most sensitive to suramin, but yeast decarboxylase is also quite sensitive (at pH 6.0) (Table 2).

Table 2 *Effect of suramin on some enzymes of yeast*

Enzyme	pH	Suramin conc	Inhibition (%)
Hexokinase	6.0-7.0	M/20,000	80-90
		M/30,000	75
Carboxylase	6.0	M/6000	68
		M/10,000	25

Suramin inhibits fumarase (Quastel, 1931) and hyaluronidase (Beiler & Martin, 1948). We have found that the following enzymes are also sensitive to the drug at pH 7.4: succinic dehydrogenase (95% inhibited by M/1000, and 75% by M/4000-suramin) and choline dehydrogenase (40% inhibited by M/5000 suramin). Amongst the enzymes not significantly inhibited by suramin at about their optimum pH's are cytochrome oxidase (pH 7.4), cholinesterase (pH 7.5), tyrosinase (pH 6.0), arginase (pH 8.9) and D-amino-acid oxidase (pH 7.4). Some of these last-named enzymes may, however, be inhibited on the acid side of their isoelectric point, for whereas catalase is not inhibited at pH 6.5 it is 90% inhibited by M/3000-suramin at pH 6.0. The specificity of these inhibitions is shown by the fact that when tests were made on the same rat-liver preparation, choline dehydrogenase was strongly inhibited (at pH 7.4), whereas D-amino acid oxidase (pH 7.4) and arginase (pH 8.9) were unaffected by suramin.

These enzyme inhibitions appear to be of two types: those at pH 7 or 7.5 (hexokinase, etc.), and the more general ones which occur only at more acid reactions. Since hexokinase and some other enzymes are strongly inhibited under physiological conditions and with suramin concentrations well within those found in the plasma after 'normal' injections of the drug, the possibility that the trypanocidal action of suramin is due partly or wholly to disturbances of the carbohydrate metabolism in the trypanosome cannot be excluded. This possibility is being investigated with living trypanosomes.

Spinks (1948) studied the retention of several suramin analogues and found that 'marked persistence is a property of polyamides of high molecular weight that contain naphthylammepolysulphonic acids as end groups'. By the courtesy of ICI (through Dr A. Spinks) we have had an opportunity of testing the enzyme inhibitory capacity of some of these analogues. We find that those suramin analogues which persist in the body for a long time after their injection also inhibit urease. It seems likely that the retention and the capacity to inhibit urease are due to interaction of the sulphonic groups of suramin and basic groups in the tissue proteins and enzyme respectively. The spatial arrangement of these sulphonic acid groups in the suramin molecule is, however, also of importance, for simple aromatic sulphonic acids do not appreciably inhibit urease.

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Some Inhibitors of Aerobic Phosphorylation By J D JUDAH and H G WILLIAMS-ASHMAN
(*Department of Morbid Anatomy, University College Hospital Medical School and Department of Biochemistry, University College, Gower Street, W C 1*)

The influence of a number of compounds on the coupling between oxidation and phosphorylation in mammalian kidney extracts has been studied. The system used contained the cyclophorase preparation of Green, Loomis & Auerbach (1948), glutamate was added as substrate, with the addition of magnesium ions, adenylic acid, fluoride, diphosphopyridine nucleotide, fructose, yeast hexokinase and inorganic orthophosphate, the pH being buffered at 7.4. All experiments were carried out at 25° C and were of short duration (6–10 min), under these conditions P/O ratios of the order of 2 were consistently obtained.

The observation of Loomis & Lipmann (1948) that low concentrations of 2,4-dinitrophenol effected a marked decrease in the rate of inorganic phosphate uptake without significantly affecting the oxygen uptake, was confirmed. When added to a final concentration of 10^{-4} M, this substance inhibited the phosphate uptake c. 90%. Somewhat higher concentrations (c. 2×10^{-4} M) of either 2-amino-4-nitrophenol or 2-nitro-4-aminophenol were devoid of significant influence on the respiration and the uptake of inorganic phosphate, as were thyroxine (10^{-4} M), thyroglobulin (4.0 mg/ml) and 10^{-3} M 4,4'-dichloro diphenyl-trichlorethane (D D T).

Marked inhibition (60–90%) of the uptake of inorganic phosphate, the respiration being either

uninfluenced or slightly stimulated, was observed in the presence of low concentrations (c. 2×10^{-5} M) of phenosafranine, thionine and janus green and somewhat higher concentrations (c. 2×10^{-4} M) of picramic acid and 2,4-dinitro-1-naphthol-7-sulphonic acid. At a concentration of 4×10^{-5} M, diethyl red, methylene blue and brilliant cresyl blue inhibited the P/O ratio 20, 38 and 40% respectively. Guanidine, methylguanidine and dimethylguanidine (10^{-3} M) were without significant influence on the P/O ratio.

At concentrations at which they inhibited the P/O ratio, all the inhibitors listed above were without influence on the activity of yeast hexokinase at 25° C.

Dinitrophenols (Dodds & Greville, 1934) and phenosafranine (Dickens, 1936) are known to be powerful inhibitors of the Pasteur effect in a number of tissues, but thionine, at concentrations comparable to those used in our experiments, has been reported to be without influence on the aerobic glycolysis of kidney (Dickens, 1934). Our experiments thus appear to cast some doubt on the suggestion of Johnson (1941) that a lowering of inorganic phosphate concentration due to aerobic phosphorylation is responsible for the aerobic inhibition of glycolysis.

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Possible Inhibitors of Muscle Phosphorylase By P N CAMPBELL (*Department of Biochemistry, University College, London, W C 1*) and N H CREASEY (*Department of Chemical Pathology, King's College Hospital, Denmark Hill, London, S E 5*)

Cori, Cori & Green (1943) have shown that glucose competitively inhibits the conversion of glucose-1-phosphate to polysaccharide with a crystalline muscle phosphorylase preparation. Cori & Cori (1940) previously showed that polysaccharide synthesis from glucose-1-phosphate with a cat liver phosphorylase preparation was also inhibited by glucose, but that mannose, galactose, maltose and fructose had no significant inhibitory effect. We have shown that these latter sugars also fail to inhibit polysaccharide synthesis from glucose-1-phosphate with the crystalline muscle phosphorylase pre-

paration of Green & Cori (1943). In addition, we have shown that the following substances also fail to inhibit the reaction with such an enzyme preparation under the conditions of our experiments: 3-methylglucose, D-xylose, 1,2-monoacetone glucose, diacetone glucose, gluconic acid, ascorbic acid, sorbitol and inositol. The concentration of the test substance in the reaction mixture was 0.05 M, except in the case of diacetone glucose when it was 0.02 M.

Cori & Cori (1940), from observations with freshly prepared glucose solutions, suggested that while α -glucose inhibited polysaccharide synthesis from

glucose 1 phosphate with their cat liver phosphorylase preparation β -glucose had little or no inhibitory effect. We have been able to confirm that an α , β specificity does exist in that solutions of α -methylglucoside significantly inhibit polysaccharide synthesis from glucose-1-phosphate with a crystalline muscle phosphorylase preparation, whereas solutions of β methylglucoside of a comparable strength show no significant inhibitory effect.

Since the reducing power of the reaction mixture

did not increase during the course of the reaction, it was concluded that under these conditions the α -methylglucoside was not hydrolyzed to glucose.

Although it is difficult to demonstrate that the inhibition by α -methylglucoside is competitive it would seem probable that this is so. These experiments suggest that the stereochemical configuration of the H atom on C₁ of the substrate is of importance for the synthesis of a polysaccharide with muscle phosphorylase.

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The Influence of Bicarbonate upon Glucuronide Synthesis By I. D. E. STOREY (Department of Biochemistry, University of Edinburgh)

In the course of a study of glucuronide synthesis by mouse liver slices, using the method of Levvy & Storey (1949), it has been observed that synthesis is considerably greater in Krebs bicarbonate Ringer than in phosphate Ringer (Table 1).

Table 2 The influence of bicarbonate upon glucuronide synthesis

Medium	Gas phase	Bicarbonate mM/l	mg o-aminophenol conjugated/g dry wt liver/hr
Phosphate Ringer	O ₂	—	0.22
Phosphate Ringer	O ₂	1.5	0.31
Phosphate Ringer	O ₂	6.0	0.37
Bicarbonate Ringer	O ₂ -5% CO ₂	24.0	0.46

Table 1 Comparison of glucuronide synthesis in bicarbonate and phosphate Ringer

Exp. no.	Mg o-aminophenol conjugated/g dry wt liver/hr				
	1	2	3	4	5
Phosphate Ringer	0.22	0.15	0.13	0.34	0.03
Bicarbonate Ringer	0.66	0.57	0.66	0.53	0.13

This effect was further investigated by adding small amounts of bicarbonate to phosphate Ringer gassed with pure oxygen (Warren, 1944), but with no provision for absorbing carbon dioxide. A typical result is shown in Table 2. The concentration of bicarbonate in the phosphate Ringer is purely nominal, since CO₂ is lost to the gas phase during the incubation period.

It is suggested that the simplest interpretation of these results is that fixation of carbon dioxide is involved in glucuronide synthesis. The observation that synthesis in phosphate Ringer without added bicarbonate was not decreased when CO₂ was absorbed by KOH is not necessarily contradictory to this suggestion, since it is known that enzyme systems concerned in fixation of carbon dioxide have a high affinity for that substance (cf. Laser, 1942).

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Iso-electric Precipitation Procedures for Purification of the Pituitary Diabetogenic FactorBy E REID (Carnegie Research Fellow) (*Department of Biochemistry, University College, London, W C 1*)

In a previous communication (Reid & Young, 1948), it was reported that diabetogenic activity was present in the precipitate obtained from alkaline saline (0.9% NaCl) extracts of ox anterior pituitary tissue, by dialysis followed by adjustment of the pH to c 6.5. The optimum pH is, in general, 6.7, if the pH 6.7 supernatant solution is adjusted to pH 6.4, the diabetogenic potency (cf Reid, 1949) of the pH 6.4 precipitate is usually lower, in terms of protein, than that of the pH 6.7 precipitate. Some extracts, however, yield only a small precipitate at pH 6.7, and it is necessary to adjust the pH to 6.6, or even 6.5, to obtain a satisfactory yield of the diabetogenic factor. An attempt has been made to correlate precipitation behaviour with factors such as (1) age of cattle from which the glands were derived, (2) volume of alkali added during extraction, and (3) period of storage of extract prior to fractionation, but the variability has not been satisfactorily explained.

Adjustment of *saline* solutions of diabetogenic precipitates to pH 5.5, or even 5.7, results in some precipitation of the factor, although Young (1938) found that precipitation did not occur when crude alkaline saline extracts were adjusted to pH 5.5. It is thought that 'inert' proteins, present in crude extracts, may modify the solubility of the factor in saline solution.

The following procedure is employed for the preparation of diabetogenic fractions. (1) The extract is adjusted to pH 5.5, and the precipitate reprecipitated from saline solution at pH 5.5. (2) The combined supernatant solutions are dialyzed at pH c 8. (3) The

solution is adjusted to pH 6.7 (or, if necessary, to pH 6.6 or 6.5), any precipitate obtained at pH 7.4 being discarded. (4) A saline solution of the precipitate, once reprecipitated from salt-free solution, is adjusted to pH 6.0 to yield a diabetogenic 'saline supernatant' fraction, which is approximately four times as pure as the crude extract in terms of protein. Typical fractions examined in the Oxford ultracentrifuge (by Mr R Cecil and Dr A G Ogston) and in the electrophoresis apparatus at the Royal Institution (by Dr P Johnson and Mr R H Smith) appeared to consist predominantly of a single protein. Further purification was attempted by ethanol precipitation procedures (cf Wilhelmi, Fishman & Russell, 1948), but it appeared, from biological tests and from ultracentrifugal examination, that ethanol did not selectively precipitate the diabetogenic factor.

Tests carried out in collaboration with Dr S J Folley have indicated that prolactin is not a major contaminant of 'saline supernatant' fractions. These fractions, in common with other pituitary preparations (cf Cotes, Reid & Young, 1949), possess growth promoting activity (assayed in intact female rats) in approximately constant proportion to the diabetogenic activity. The tentative conclusion that the diabetogenic activity of these fractions could be attributed to growth hormone is supported by the ultracentrifuge data, the observed sedimentation constants being similar to those recently reported for pure growth hormone (Smith, Brown, Fishman & Wilhelmi, 1949).

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The Influence of Insulin and Anterior Pituitary Factors on the Hexokinase Activity of Animal Tissue Extracts By R H SMITH* (introduced by F G YOUNG) (*Department of Biochemistry, University College, London, W C 1*)

C F Cori and his school (e.g. Cori, 1945-6, Colowick, Cori, G T & Slein, 1947) have recently directed attention to hexokinase as one possible locus of action of insulin and factor(s) of the anterior hypo-

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physis. These authors have reported that, *inter alia* (a) The hexokinase activity of freshly prepared extracts of muscle from alloxan-diabetic rats may be markedly elevated by the addition of insulin to the test system *in vitro*. (b) The hexokinase activity of extracts of brain or muscle from normal animals may

be inhibited by the addition of certain anterior pituitary preparations *in vitro*, this inhibition may be counteracted by the addition of insulin to the system *in vitro*

Studies have been made on the influence of insulin on the hexokinase activity of freshly prepared extracts of skeletal muscle from alloxan-diabetic rats. Assays of hexokinase activity were made exactly as described by Colowick *et al* (1947), 0.2 ml of adrenal cortical extract ('Eucortone', Allen and Hanbury) was added to each test system. From a total of twelve rats made acutely diabetic by treatment with alloxan only one animal afforded a muscle extract that showed a pronounced elevation of hexokinase activity upon the addition of insulin *in vitro*. This finding is in marked contrast to that of Colowick *et al* (1947), who reported that, from a total of thirty alloxan diabetic rats, fifteen afforded muscle extracts which, in the presence of adrenal cortical extract, showed elevations of hexokinase activity of from 24 to 79% upon the addition of insulin to the test system. A similar failure of insulin to elevate the hexokinase activity of extracts of muscle from alloxan-diabetic rats was reported by

Broh-Kahn & Mirsky (1947), these authors, however, did not carry out the assays in the presence of adrenal cortical extract. Recently, Stadie & Hangaard (1949) have also reported *inter alia* the failure to observe a significant effect of insulin upon the hexokinase activity of alloxan-diabetic rat muscle extracts in the presence of adrenal cortical extract.

Confirmation of the second observation of the Cori school has been reported by Broh-Kahn & Mirsky (1947) and by Reid, Smith & Young (1948). Nevertheless, both groups of authors pointed out the wide divergence between the diabetogenic activity and the insulin reversible hexokinase inhibitory activity of anterior pituitary extracts. This divergence has again been emphasized by recent studies with highly purified diabetogenic fractions of ox-pituitary extract prepared by Mr E. Reid (Reid, 1949). A number of these preparations, which were highly diabetogenic in the intact adult cat, have been tested for hexokinase inhibiting activity following Colowick *et al* (1947), in no instance has such a preparation been found to inhibit the activity of rat brain hexokinase *in vitro* in a manner reversible by insulin.

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DEMONSTRATIONS

Application of Paper Partition Chromatography to Metabolic Studies with Radioactive Iodine
By J. C. LAIDLAW (introduced by F. G. YOUNG) (*Department of Biochemistry, University College, London*)

Chromatography of Vitamin A and Derivatives on Alumina-treated Filter Paper By S. P. DATTA and B. G. OVERELL (*Department of Biochemistry, University College, London*)

Small amounts of vitamin A, its esters, anhydro-vitamin A, retinene and other chromogens may be distinguished by chromatography on filter paper impregnated with alumina. Whatman no. 54 filter papers are dipped in a solution of aluminium sulphate (65 g. $\text{Al}_2(\text{SO}_4)_3$ per l.), the excess fluid is allowed to drain and the papers are dipped in 2 N-ammonia and placed in running water for 5 hr. The papers are dried at 90° C. The chromatogram is run with light petroleum (80-100° C.) in an atmosphere

of nitrogen, and rapidly sprayed with the Carr-Price reagent to develop the colour. If the papers are rigorously standardized constant R_f values are obtainable. It is, however, convenient to run a marker of vitamin A ester, or of the dyes Sudan yellow and Sudan red with each run, from which a measure of the activity of the paper is obtained. Amounts of vitamin A down to 1 μg (c. 3 i.u.) may be readily identified by this method.

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*

Displacement chromatography on synthetic ion exchange resins 2 The separation of organic acids and acidic amino acids by the use of anion exchange resins By S M PARTRIDGE and R C BRIMLEY

Displacement chromatography on synthetic ion exchange resins 3 Fractionation of a protein hydrolysate By S M PARTRIDGE

The distribution of methane in animal tissues, as determined by a merodiffusion method, and the effect of urethane treatment on enzymes By E BOYLAND and EVA RHODIN

An immune globulin fraction from bovine precolostrum By E I McDougall

A further study of hydrolysates of grameidin By R L M SYNGE

A study of the acidic peptides formed on the partial acid hydrolysis of wool By R CONSDEN, A H GORDON and A J P MARTIN

Rancidity in Indian butterfats (ghee) By K T ACHALA

The use of the tyrosine apodecarboxylase of *Streptococcus faecalis* R for the estimation of codecarboxylase By G H SLOANE STANLEY

A quantitative study of complex formation in heated protein mixtures By A KLECZKOWSKI

Nitrogenous excretion in Chelonian reptiles By VIVLEN MOYLE

The concentration and distribution of haemoglobin in the root nodules of leguminous plants By J D SMITH

Haemoglobin and the oxygen uptake of leguminous root nodules By J D SMITH

The inhibition of β glucuronidase by saccharic acid and the role of the enzyme in glucuronide synthesis By M C KARUNAIRATNAM and G A LEVY

The metabolism of chrysene the isolation of 3-methoxychrysene by methylation of the phenolic metabolite of chrysene from rat faeces By I BERENBLUM and R SCHOENTAL

Bile acid enteroliths, with an account of a recent case By F S FOWWEATHER

The Bence Jones protein of multiple myelomatosis its methionine content and its possible significance in relation to the aetiology of the disease By C E DENT and G A ROSE

The fate of certain organic acids and amides in the rabbit 7 An amidase of rabbit liver By H G BRAY, SYBIL P JAMES, ISABEL M RAFFAN, BRENDA E RYMAN and W V THORPE

The enzymic hydrolysis of glutamine and its spontaneous decomposition in buffer solutions By H G BRAY, SYBIL P JAMES, ISABEL M RAFFAN and W V THORPE

The effect of various diets on the metabolism of nicotinic acid and nicotinamide in the rabbit By P ELLINGER and M. M ABDEL KADER

Regulation of urinary steroid excretion 1 Effects of dehydroisandrosterone and of anterior pituitary extract on the pattern of daily excretion in man By M REISS, R E HEMPHILL, J J GORDON and E R COOK

The use of the Waring blender in biochemical work By ROSA STERN and L H BIRD

A mercury gasometer for the preparation of gas mixtures By E F HARTREE and C H HARPLEY

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 276th Meeting of the Society was held in the Department of Biochemistry, University Museum, Oxford, on Saturday, 7 May 1949, when the following papers were read

COMMUNICATIONS

Effect of Glucose on the Utilization of Acetate by Lactating Mammary Gland Slices By S. J. FOLLEY and T. H. FRENCH (*National Institute for Research in Dairying, University of Reading*)

Hitherto, the utilization of acetate for fat synthesis *in vitro* has been demonstrated only with tracers, since liver, believed to be most active in this respect, does not effect net synthesis outside the body. However, lactating mammary gland slices from ruminants (cow, goat) markedly utilize acetate with high R.Q.

studied the effect of glucose on the utilization of acetate by lactating rabbit mammary gland slices with results summarized below. The methods were as previously used (Folley & French, 1948).

Addition of glucose increased Q_{O_2} and markedly raised the acetate uptake over the trivial values

(0.02M acetate
+ 0.1% glucose) -
(0.1% glucose)

								Mean
$\Delta - Q_{O_2}$	4.5	4.8	-0.2	4.8	2.3	4.1	4.6	3.6
$\Delta Q_{O_2}^{acid}$	-7.1	-8.1	-3.7	-5.8	-5.6	-5.0	-6.8	-6.0
$\Delta R.Q.$	-0.03	-0.08	0.25	0.26	0.22	-0.26	-0.13	0.03

but not glucose. On the other hand, slices from non-ruminants (rat, mouse, rabbit, guinea pig) which readily metabolize glucose with high R.Q. are almost inert towards acetate (Folley & French, 1948). The results with ruminant slices, now extended to sheep, were interpreted as indicating acetate utilization for milk fat synthesis.

The failure of non-ruminant mammary slices to utilize acetate *in vitro* is rather puzzling in view of Popjak & Beeckmans' (1949) finding that glyceride fatty acids isolated from the mammae of pregnant rabbits fed labelled acetate contained a high concentration of ^{14}C . These results, and our subsequent finding in collaboration with Dr G. Popjak that the ^{14}C is particularly concentrated in the lower (volatile) fatty acid fraction, clearly point to acetate utilization for milk fat formation in the rabbit.

Following Bloch & Kramer's (1948) demonstration that pyruvate or glucose increases acetate incorporation into fatty acids by liver slices, we have

obtained with acetate alone R.Q. (always > 1) was increased in three experiments, hardly altered in two, and decreased in two. The ratios of extra acetate to O_2 were large enough virtually to exclude the possibility that the former was merely oxidized, so acetate must have been incorporated into fatty acids as indicated by the maintenance of the R.Q. above unity. Glucose may act by providing glycerol for glyceride synthesis.

In four preliminary experiments on rabbit mammary slices and six on rat, we have found that these effects of glucose are further increased by insulin, the hormone giving the following mean increases (rabbit) over control values: $-Q_{O_2}$, 1.3; $Q_{O_2}^{acid}$, -3.1; R.Q., 0.40.

Bloch & Kramer reported an insulin effect with pyruvate but not glucose. The apparent difference from our results is explained by our finding that *o*-cresol, present in our insulin preparation, increases glycolysis thus providing lactate or pyruvate on which insulin can act.

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Investigations on Porphyrin Formation in Congenital Porphyrria with the aid of ^{15}N By C. H. GRAHAM and A. NEUBERGER (*Department of Chemical Pathology, King's College Hospital, London, S.E. 5 and the National Institute for Medical Research, Hampstead, N.W. 3*)

Twelve grams of glycine containing 31.65 atom % excess ^{15}N was fed in 12 doses over 4 days to a patient (G.L.) suffering from congenital porphyrria. The faecal and urinary coproporphyrin, the urinary uro-

porphyrin and stercobilin hydrochloride were isolated and the abundance of ^{15}N determined with the mass spectrometer. Haemin was also isolated. The results are shown in Table 1.

Table 1 Atom percentage excess ^{15}N formed in the various porphyrin fractions and in stercobilin after feeding 12 g of glycine containing 31.65 atom % excess ^{15}N to a case of congenital porphyria

Days of exp	^{15}N atom % excess in			
	Urinary copro porphyrin	Faecal copro porphyrin	Urinary uro porphyrin	Stercobilin hydro chloride
1-4	2.23	1.07	1.165	1.159
5-8	1.1	2.24	1.46	1.30
9-12	0.499	0.78	0.85	1.36
13-16	—	0.401	—	0.531
17-20	—	0.243	—	0.303
21-24	—	0.177	—	0.220
25-28	—	0.128	—	0.150

The high ^{15}N content of the excreted porphyrins indicates that the nitrogen of both uro- and coproporphyrin I like that of protoporphyrin IX of haemin is derived, probably exclusively, from glycine. The

^{15}N content in urinary uroporphyrin rises sooner than that of faecal coproporphyrin but does not reach the same high values. Most remarkable is the high ^{15}N content of stercobilin which indicates that most of this pigment is derived by some pathway other than by breakdown of haemoglobin. This holds for the normal person, in which glycine containing ^{15}N is also incorporated rapidly into stercobilin, but the concentration of the isotope is then very much less than in the case of congenital porphyria.

The patient was also given daily 2 g of sodium benzoate for the first 12 days of the experiment and the ^{15}N content of the excreted hippuric acid was estimated. The results indicate that the glycine used for hippuric acid synthesis has a lower isotope content than the precursor of the porphyrins. It appears that the glycine required for the conjugation with benzoic acid is partly obtained by hydrolysis of tissue protein or is synthesized from nitrogenous compounds with a low isotope content.

In vivo Formation of Citrate By P. BUFFA and R. A. PETERS (Department of Biochemistry, University of Oxford)

The Specificity of Pigeon-Brain Cholinesterase By V. P. WHITTAKER (Department of Biochemistry, University of Oxford)

Following Bodansky's (1946) observation that brain and erythrocyte cholinesterase would hydrolyze triacetin, Adams & Whittaker (1948) (full report by Adams, 1949) investigated the specificity of purified human erythrocyte cholinesterase and found that it would hydrolyze a wide range of aliphatic esters. The rate of hydrolysis was found to depend on (a) the size of the acyl group, acetate being optimal, and (b) the configuration of the alkyl group, 3,3-dimethyl butyl acetate, the carbon analogue of acetyl-choline being most rapidly split and other alkyl esters being less rapidly split the greater their deviation from this configuration.

It has been known for some years that there is a close similarity between the cholinesterases of brain and erythrocytes, and it was therefore of interest to see whether the erythrocyte specificity pattern was also shown by the brain enzyme. Pigeon brain was selected for this purpose as it has a high cholinesterase activity per unit weight. It has now been found that the specificity of the pigeon brain enzyme closely resembles that of the erythrocyte enzyme, except that the rates of hydrolysis of the aliphatic esters relative to acetyl- β -methyl choline are one half to three quarters of the values obtained with the erythrocyte enzyme (Fig. 1).

Pigeon brain, like human erythrocytes, contains a small quantity of alsesterase which it is necessary

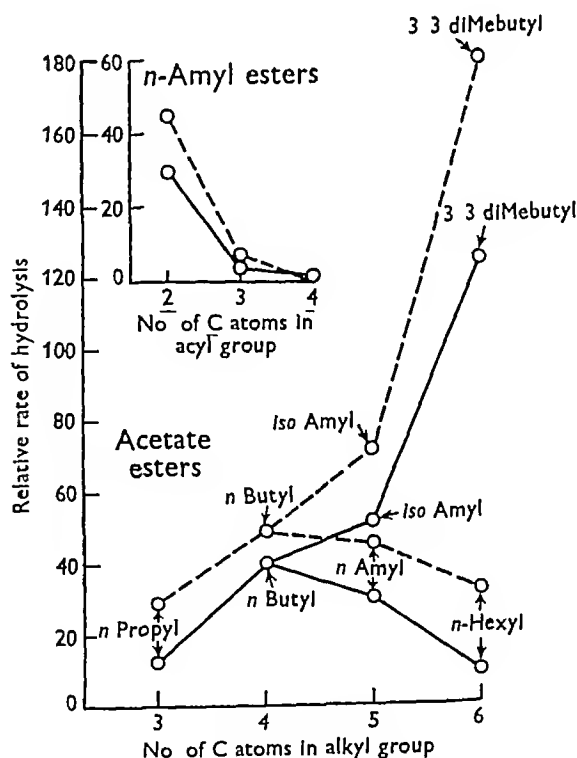


Fig. 1 Rate of hydrolysis of aliphatic substrates by pigeon brain cholinesterase (o—o), and human erythrocyte cholinesterase (o---o)

to remove when studying the aliphatic esterase activity of the cholinesterase. This has been achieved by differential centrifuging and washing of the fraction precipitating between 4500 and 15,000 r.p.m. Inhibitor and summation experiments confirmed the homogeneity of the purified fraction. The degree of

purification and the yield are not high and the enzyme appears to be adsorbed on to particles of very varying size. It is only weakly adsorbed on to acid-washed kieselguhr at pH 6.5. Its physical properties are thus somewhat different from those of the erythrocyte enzyme.

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The Effect of Stimulation on the Phosphate Esters of the Brain By D. RICHTER and R. M. C. DAWSON (Beit Memorial Fellow) (*Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff*)

The sequence of changes in the rat brain after stimulation was studied by determining the phosphate esters in brains taken at varying times during and after electrical stimulation. In order to fix the changes in the tissue, the brain was rapidly frozen *in situ* with liquid air.

Electrical stimulation produced (a) a rapid fall in the phosphocreatine level, and (b) a corresponding rapid but transient rise in the hexose phosphate fraction. These changes were attributable to the phosphorylation of glucose. Over 50% of the total phosphocreatine of the brain was lost after 1 sec. of electrical stimulation.

The initial changes were followed by (c) the return of the hexose phosphate fraction to the normal level, accompanied by (d) the liberation of inorganic phosphate. These changes were evident in brains fixed up to 10 sec. after electrical stimulation; they occurred before the onset of convulsions.

Brains fixed at 15–45 sec. after electrical stimulation showed (e) a return of the phosphocreatine and (f) of the inorganic level to normal. The rate of re-synthesis of phosphocreatine under these conditions, as calculated from the slope of the curve, was 1.25 mg./g. fresh brain tissue per min. The adenosine triphosphate fraction showed only a slight fall after stimulation, with a return to normal after about 25 sec. The brains of rats taken during emotional excitement gave a mean phosphocreatine level 13% higher than that of normal controls; this indicated that the re-synthesis of phosphocreatine was favoured under these conditions.

The method of fractionation and estimation of the phosphate esters was based on that of Stone (1943). Stone, Webster & Gurdjian (1945) have previously described changes in the phosphate esters of the brain as a result of stimulation.

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The Influence of Dietary Bromobenzene and L-Cystine on the Bromine Content of Rat Hair
 By B. SPENCER and R. T. WILLIAMS (*Departments of Biochemistry, University of Liverpool and St Mary's Hospital Medical School, University of London*)

Our object was to find out how orally administered compounds could be made to appear in the hair or skin. Compounds which might appear in the hair are those known to conjugate with cysteine. A study was therefore made of the effect of feeding varying amounts of L-cystine and bromobenzene on the bromine content of the hair of albino rats. Six groups of four rats (♀) were kept on a normally adequate diet. At the beginning of the experiment

the hair of all rats was removed under anaesthesia with electric clippers. Groups I–V received PhBr and groups I–IV, L-cystine. Groups VI were controls receiving no PhBr or cystine. Urine was collected daily and pooled 3-day urines analyzed for *p*-bromophenylmercapturic acid. After 30 days, three rats in each group were killed and the hair, liver and thigh muscles analyzed for Br, and the hair for cystine. The 4th rat in each group was kept for another

Table 1

Group	Added to diet (g /100 g)		Cystine content of hair on 30th day	Percentage PhBr excreted as mercapturic acid	Weight, gain or loss on 30th day
	L-Cystine	Bromobenzene			
I	0.2	0.5	13.13	27	Slight gain
II	0.4	1.0	13.18	31	Slight gain
III	0.7	1.5	13.25	29	Slight gain
IV	1.0	2.0	13.28	28	Slight loss
V	0	1.5	12.29	14	Loss
VI (controls)	0	0	13.16	—	Slight gain

Table 2

Group	Bromine content of tissues ($\mu\text{g/g}$)					
	On 30th day			On 44th day		
	Hair	Muscle	Liver	Hair	Muscle	Liver
I	149	135	108	165	71	58
II	178	122	165	206	59	69
III	174	147	151	214	74	71
IV	236	173	168	295	93	78
V	605	112	101	702	54	64
VI (controls)	49	28	35	47	27	37

14 days, but during this period received no PhBr or extra cystine. On the 44th day these were killed and the tissues analyzed.

The following points are to be noted:

(a) The cystine content of the hair of group V suggests cystine insufficiency, whereas groups I–IV and VI are normal.

(b) Group V produce less mercapturic acid than the others.

(c) The Br contents of the hair, muscle and liver of groups I–IV are of the same order, but are higher than controls.

(d) The Br content of the hair of group V is much higher than other groups, whereas the Br content of

muscle and liver of group V is similar to other groups.

(e) Withdrawal of PhBr results in a drop in muscle and liver Br, but hair Br remains high in all groups.

The results suggest that Br appears in the hair in relatively large amounts when the cystine of the diet is insufficient to cover the needs of both detoxication and normal requirements. It is possible that the cystine of the hair is slightly replaced by *p*-bromophenylcysteine, but this has not yet been proved. If this were so the hair of group V would contain about 210 mg % of *p*-bromophenylcysteine on the 30th day and about 240 mg % on the 44th day.

Mechanism of the Aerobic Oxidation of Reduced Cozymase by Heart Muscle By E. C. SLATER (Australian National University Fellow) (Molteno Institute, University of Cambridge)

While it is generally accepted that the aerobic oxidation of reduced cozymase (CoH_2) by tissues requires diaphorase and cytochrome oxidase, there is no agreement concerning the components of the cytochrome system involved (Dewan & Green, 1938, Lockhart & Potter, 1941, Okuniki & Yakushiji, 1940).

CoH_2 , added to Keilin & Hartree's (1947) heart-muscle preparation, under anaerobic conditions, immediately and completely reduced cytochromes *c* and *a*. A very faint *b* band appeared shortly afterwards. The bands disappeared immediately on admitting air. It is concluded that cytochromes *c* and *a*, but not *b*, are directly involved in the oxidation of CoH_2 .

The rate of oxidation of CoH_2 by heart-muscle

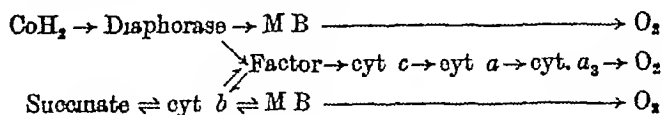
preparation was found, by measurement of the rate of the decrease of the optical density at $340\text{ m}\mu$ in a Beckmann spectrophotometer, to be equivalent to a Q_{O_2} ($\mu\text{l O}_2/\text{mg fat-free dry wt./hr.}$) of 135 at 20° and pH 7.3. This is about the same as the succinic oxidase activity of this preparation. The oxidation of CoH_2 was almost completely inhibited by cyanide and azide and restored again by the addition of methylene blue. The rate was decreased to 5 % of the control by shaking the heart-muscle preparation with BAL, in air, at 19° under conditions which caused the complete inactivation of the succinic oxidase system. Treatment with BAL did not affect the oxidation of CoH_2 by the heart-muscle preparation in the presence of azide and methylene blue. BAL, in the absence of air, had no effect. The most probable

explanation of these findings is that the oxidation of CoH_2 requires the same factor which has previously been shown to be required for the reduction of cytochrome *c* by cytochrome *b* in the succinic oxidase system, and which is destroyed by certain reducing agents in the presence of air (Slater, 1948, 1949)

CoH_2 was oxidized anaerobically by fumarate in the presence of the heart muscle preparation at a rate 1-2% of that of the aerobic oxidation of CoH_2 . This anaerobic reaction was inhibited by malonate and by treating the preparation with BAL in air

The faint *b* band, which appeared on adding CoH_2 to the preparation under anaerobic conditions, disappeared on adding fumarate

On the basis of these experiments, the coenzyme and succinic oxidase systems may be formulated as follows, the arrows indicating the direction of hydrogen or electron transfer



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Non-utilization of Labelled CO_2 and Formate for the *in vitro* Synthesis of Haem by Surviving Fowl Erythrocytes By A W J BUFTON, R BENTLEY and C RIMINGTON (*Department of Chemical Pathology, University College Hospital Medical School, W C 1, and the National Institute for Medical Research, Hampstead, N W 3*)

The nitrogen atoms of haemin are known to be derived from glycine (Shemin & Rittenberg, 1946) and the methylene carbon atom of this amino acid (Altman, Casarett, Masters, Noonan & Salomon, 1948), but not the carboxyl carbon atom (Grinstein, Kamen & Moore, 1948), is also specifically utilized in the haemin synthesis. Bloch & Rittenberg (1945) earlier presented evidence that significant amounts of deuterium are incorporated into the haemin of rats fed deuterioacetate. Apart from this, nothing is known with certainty concerning the precursors of the carbon atoms of the haemin molecule.

Recently, Armstrong, Schubert & Lindenbaum (1948) implanted a tablet of $\text{Ca}^{14}\text{CO}_3$ into a rat, and from the finding that ^{14}C could be detected in the blood haemin, concluded that there was a significant incorporation of ^{14}C into this substance. In view of the low activity of the haemin obtained in this experiment, such an inference seemed to us open to question, and we have therefore sought direct evidence by another method.

Shemin, London & Rittenberg (1948) have shown that the nucleated erythrocytes of ducks' blood will continue to synthesize haemin *in vitro* from labelled glycine. We have used chicken blood which we find behaves in a similar manner. Neither $^{13}\text{CO}_2$, constituting 5% by volume of the gas phase above the cells, nor labelled formate added in the proportion of 25 mg of $\text{H}^{13}\text{COONa}$ to 2.5 ml of blood was utilized for haemin synthesis during 24 hr at 37° . We believe, therefore, that the incorporation observed by Armstrong *et al* (1948) was due rather to a general labelling of metabolic intermediates than to a direct specific utilization.

Assuming that the surviving fowl erythrocyte is capable of carrying out the CO_2 fixation reactions which have been demonstrated with many other types of cell, we may argue that molecules such as pyruvate, oxalacetate, ketoglutarate, etc., are unlikely to be precursors of the carbon atoms of the haemin molecule synthesized by this system.

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N-(α -naphthyl)-ethylenediamine The P A S concentration is determined separately by the *p*-dimethylaminobenzaldehyde method and then, from standard curves of sulphetrone and P A S prepared by the diazotization method, a correction for the P A S concentration may be made and the

concentration of sulphetrone in the presence of P A S calculated

Mixtures of P A S and sulphetrone added to blood gave recoveries of 94–110 % for P A S and 90–116 % for sulphetrone

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Studies on a Bacterium needing Long-Chain Unsaturated Fatty Acids for Growth By M R POLLOCK, G H HOWARD and B W BOUGHTON (*Medical Research Council Unit for Bacterial Chemistry, Lister Institute, London, S W 1*)

A diphtheroid bacterium (provisionally labelled '*Corynebacterium Q*') isolated as a chance contaminant in the laboratory was found to grow very poorly on ordinary tryptic meat agar with or without blood. In the presence of 1/25,000 oleic acid, however, growth was luxuriant. Further experiments in a chemically defined medium containing 18 amino-acids and 16 growth factors showed that its need for oleic acid was absolute, and the total yield of cells was directly proportional to the amount of oleate added, between concentrations of 0.5 and 10 μ g/ml.

Elaidic, petroselinic, linoleic, linolenic and palmitoleic acids could each replace oleic acid almost quantitatively, although the last three were inhibitory in higher concentrations. $\alpha\beta$ Oleic, ricinoleic, erucic and brassidic acids were all inactive. All saturated fatty acids tested (lauric, myristic, palmitic, stearic, dibromostearic and dihydroxystearic acids) were also quite inactive.

Thus, this organism requires a fatty acid containing at least one double bond. Activity is still present when there is more than one double bond (linoleic or linolenic acids) though it is abolished by a hydroxyl group (ricinoleic acid). The position of the double bond relative to the carboxyl group is not highly specific since petroselinic acid (6/7) is nearly as satisfactory as oleic acid (9/10). The 2/3 isomer ($\alpha\beta$ -oleic acid), however, is inactive. The configuration

around the double bond is not critical, since elaidic acid is as active as its *cis* isomer (oleic acid). It is also possible to remove 2 (CH_2) groups from the end of the oleic acid molecule (forming palmitoleic acid) without greatly affecting the activity.

The relationship between total growth and concentration of oleate is sufficiently constant under appropriate conditions, to allow a fairly accurate assay of this compound in very small quantities ($\text{SD} \pm 5\%$ between 2.0 and 10 μ g/ml), but the rather wide limits of specificity inevitably reduce its value as an assay organism for oleic and other unsaturated fatty acids in biological extracts.

The essential nature of certain unsaturated fatty acids for the proper nutrition of a variety of organisms, ranging from mammals and insects to fungi and bacteria, is now fairly well established. However, so little is known about the functions of lipoids in micro organisms that it is impossible to decide whether these essential fatty acids are incorporated into the cell as such, or form some specific source of energy or merely have some physico-chemical action—e.g. at the cell surface. It is hoped, however, that microanalysis of the lipid constituents of cells of '*Corynebacterium Q*' grown in a synthetic medium with the various essential fatty acids mentioned above (in particular the 'unnatural' elaidic acid) may throw some light on this problem.

Metabolic Studies with 3-Methylglucose By P N CAMPBELL * (*Department of Biochemistry, University College, London, W C 1*)

Since the chemical and physical properties of 3-methylglucose closely resemble those of glucose it was of interest to determine how the introduction of

a methoxyl group into the glucose molecule on C_3 affected its metabolism in the body.

For this purpose a 50 % aqueous solution of 3-methylglucose was fed to starving rats by stomach tube. Control experiments under similar conditions

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were carried out in which a 50 % aqueous solution of glucose or, alternatively, water was fed. After a 3 hr absorption period the rats were killed and the glycogen content of the liver and carcass determined. The methoxyl content of the glycogens and their acid hydrolysates was estimated. Estimations were also carried out to determine the extent of the absorption of the sugar from the intestine and its concentration in the blood and in the urine. The results showed that the liver and carcass glycogen level in the rats fed 3-methylglucose was not significantly different from that of the rats which only received water. The methoxyl content of the acid hydrolysates of the glycogens of the 3-methylglucose-fed rats was not significantly greater than that from the other groups. These results suggest that 3-methylglucose is not converted to glycogen in the rat and that a methylated polysaccharide is not formed. The estimations on the gut contents after the absorption period showed that not more than 10–15 % of the sugar remained unabsorbed. More extensive studies on this point have previously been reported (Campbell & Davson, 1948). A reducing sugar was found in the urine.

There was no increase in the methanol content of

the urine of rats fed 3-methylglucose. When 3-methylglucose was given as a 6 % solution by intra-peritoneal injection unchanged sugar in the urine was demonstrated by the preparation of an osazone, the crystal structure of which closely resembled that of 3-methylglucosazone. An average of 92 % of the administered sugar was found in the urine. It is concluded that not more than about 10 % of the administered 3-methylglucose can be metabolized in the rat.

By estimating the loss of reducing power of a glucose solution during fermentation with baker's yeast in the presence and absence of 3-methylglucose, it was found that the latter did not affect the rate of fermentation of glucose by yeast. Similarly 3-methylglucose failed to inhibit the synthesis of a polysaccharide from glucose-1-phosphate with a crystalline muscle phosphorylase preparation, in contrast with glucose (Cori, Cori & Green, 1943, Campbell & Creasey, 1949).

These experiments show that the introduction of a methoxyl group into the glucose molecule at C₃ profoundly affects the metabolism of the substance in the animal body.

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The Effect of Cocarboxylase and of Oxygen on the Formation of Citrate and α -Ketoglutarate by Pigeon-Brain Homogenates. By R. V. COXON (Betty Brookes Fellow) and R. A. PETERS (Department of Biochemistry, University of Oxford)

It has been suggested (cf. Wood, Werkman, Hemingway & Nier, 1942) that a 7-carbon compound may be an initial stage in the formation of citrate from the condensation of pyruvate and oxaloacetate in animal systems, rather than a 6-carbon acid arising by the combination of an active 2-carbon derivative of pyruvate with the oxaloacetate. Using dialyzed homogenates ('dispersions') of brains obtained from healthy pigeons as in previous work (Coxon, Liébecq & Peters, 1949) we have found that these

preparations form more citrate when incubated in an atmosphere of air than in nitrogen. If dispersions from B₁-avitaminous birds are employed, the addition of cocarboxylase *in vitro* induces a further increase in citrate production, while a similar relationship holds also for α -ketoglutarate. This evidence supports the view that, in this system, oxidative decarboxylation with the formation of a 2-carbon fragment is a definite step in the metabolism of pyruvate.

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Quantitative Paper Partition Chromatography By R B FISHER and R HOLMES (*Department of Biochemistry, University of Oxford*)

The logarithmic relation between the area of the coloured spot on the one-dimensional paper partition chromatogram and the amount of the particular substance placed on the paper which was described by Fisher, Parsons & Morrison (1948) has been made the basis of a method of assay of amino acids. The assay procedure involves simultaneous development, on the same paper, of chromatograms of four samples of equal volume, two of the unknown and two of standard solutions, the ratio of the concentrations of the two unknowns being the same as the ratio

of the concentrations of the two standards. From the areas of the coloured spots produced the log of the ratio of concentrations of corresponding standard and unknown solutions can be obtained without prior calibration. With adequate replication the range of error in the present state of the method is $\pm 5\%$, when applied to samples containing 1–2 μg NH_3N .

A rapid photoelectric method of spot area measurement is described.

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Paper Chromatography of Liver Extracts By G E SHAW (*Evans Biological Institute, Runcorn, Cheshire*)

Cuthbertson & Lester Smith (1949) described a method of applying paper chromatography to liver extracts. They developed the chromatogram with butanol saturated with water, and demonstrated several growth factors for *Lactobacillus lactis* Dorner by an impress technique on agar plates. The present investigation is concerned with the quantitative aspect of paper chromatography as applied to liver extracts. A quantity of liver extract corresponding to about 0.05 μg B_{12} in 0.005 ml is placed on a strip of no. 1 Whatman filter paper 1 cm wide. The strip is suspended in a glass cylinder containing butanol saturated with water, the whole protected from light, allowed to develop for a suitable period by capillary migration, coupled with evaporation at the advance front. The strip is then cut into suitable lengths, these sections are extracted with water and the extracts assayed for B_{12} activity. To determine the proportion of total Dorner activity which migrates at a speed greater than that of riboflavin, a spot of riboflavin is placed under the liver extract spot and developed for 48 hr. The riboflavin will then have migrated to an extent of about 6 cm, whereas the advance front will be about 13 cm from the initial spot. The strip is bisected at the riboflavin belt. The volume of water used to extract the sections depends on the amount of B_{12} activity expected for

each section which is determined by preliminary titration. For a complete 'spectrum' it is necessary, under the conditions in these laboratories, to develop the strip with butanol for 14 days or even longer, when almost all activity will have left the spot, a sharp peak found at a distance of about 3 cm with the advance front about 13 cm. The strip is dried and cut into portions 0.5 cm long. The liquid medium is used for assay. The basal medium consisting of acid hydrolyzed casein, 0.1% Tween 80, the usual salts and vitamins as for *Lb. helveticus*, is autoclaved at pH 5.5. Glucose, T J supplement and vitamin B_{12} preparation are added aseptically. The inoculum is a dilute suspension (opacity one-fifth) of young activity growing *Lb. lactis* Dorner. Incubation is for 16 hr, after which the opacity is read on a Spekker. The T J supplement used is a concentrate of the fraction of potato extract soluble in 50% but insoluble in 80% ethanol, which gives a very low 'blank', the amount per tube is 1–2000 on final medium. Summation of the amounts of B_{12} activity in each individual section show fairly good recovery of the total amount placed on the spot. For the majority of liver extracts examined, no significant percentage of total activity has been found to migrate faster than riboflavin.

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DEMONSTRATION

Isolated Nuclei from Cells of the Cerebral Cortex By D. RICHTER and R. P. HULLIN (*Neuro-psychiatric Research Centre, Whitchurch Hospital, Cardiff*)

A method is given for the preparation in quantity of the nuclei of the cells of the cerebral cortex. The method depends on repeated differential centrifugation at varying speeds after breaking up the cells by grinding with ice in dilute citric acid solution (100 g tissue, 200 g ice, 20 ml 1.1% citric acid solution). It is similar to the procedure described by Dounce (1943) for the isolation of nuclei from liver cells, but which Mirsky & Pollister (1946) found inapplicable to nervous tissue.

The method now described has been applied to the rabbit brain and to fresh human post-mortem material. It is mild enough to leave most of the enzymes of the tissue intact, and it is therefore applicable to studies of the distribution of enzymes and other labile substances in the cell. Nuclei from the human cerebral cortex were found to contain a particularly active alkaline phosphatase, which is present in higher concentration in the nuclei than in the cytoplasm of the cell.

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FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*

- A comparative study of the succinic dehydrogenase cytochrome system in heart muscle and in kidney By E C SLATER
- The action of inhibitors on the system of enzymes which catalyse the aerobic oxidation of succinate By E C SLATER
- A respiratory catalyst required for the reduction of cytochrome *c* by cytochrome *b* By E C SLATER
- Nitrogen in human dental enamel By D J ANDERSON
- Interrelationship of certain vitamins of the B group in aneurin, riboflavin and biotin deficiencies By K BHAGVAT and P DEVI
- The fractionation of weak electrolyte mixtures by ion exchange resins By C W DAVIES
- Direct transformation of fumarate to oxaloacetate, without intermediate formation of malate, by *Clostridium saccharobutyricum*, strain GR4 By GERMAINE COHEN-BAZIRE and G N COHEN
- The fate of certain organic acids and amides in the rabbit 8 Toluic acid and amides By H G BRAY, W V THORPE and P B WOOD
- Properties of the acid phosphatases of erythrocytes and of the human prostate gland By M. A. M. ABUL FADL and E J KING
- The micro and ultramicro determination of chlorine in organic compounds By J D JUDAH
- Studies in detoxication 25 The characterization of phenylglucuronide, and its rate of hydrolysis compared with that of phenylsulphuric acid By G A GARTON, D ROBINSON and R T WILLIAMS
- Some effects of glucose and calcium upon the metabolism of kidney slices from adult and newborn rats By J R ROBINSON
- A new manometric method for determination of respiratory quotients By H LASER and LORD ROTHSCILD
- The estimation of trichloroethylene in blood By F H BRAIN and P J HELLIWELL
- Vitamin P III By S S ZILVA
- The micro estimation and origin of methylamine in *Mercurialis perennis* L By B T CROMWELL
- The metabolism of the amino sugars I The breakdown of *N* acetylglucosamine by strains of *Streptococcus haemolyticus* and other streptococci By H J ROGERS
- The kinetics and specificities of deamination of nitrogenous compounds by X-radiation By W M DALE, J V DAVIES and C W GILBERT
- An investigation of the intracellular fluid of calf embryo muscle By A H GORDON
- The effect of dietary oleic and palmitic acid on the composition and turnover rates of liver phospholipids By I G CAMPBELL, JUNE OLLEY and M BLEWETT
- Renal function as affected by experimental unilateral kidney lesions 2 The effect of cyanide By T F NICHOLSON

